



# Antibiotics in the environment : impact of metal oxides and effect on denitrification

Chen Chen

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# THESE DE DOCTORAT DE

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Par

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« Antibiotics in the environment: impact of metal oxides and effect on denitrification »

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# List of Publications

**Chen Chen**, Anniet M. Laverman, Céline Roose-Amsaleg, Garance Regimbeau, Khalil Hanna. Fate and transport of tetracycline and ciprofloxacin and impact on nitrate reduction activity in coastal sediments from the Seine Estuary, France. *Environmental Science and Pollution Research*, 2023, 30(3): 5749-5757.

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**Chen Chen**, Céline Roose-Amsaleg, Anniet M. Laverman. Antimicrobial resistance in the riverine denitrifying *Pseudomonas veronii*. (In preparation).



# Abstract

Antibiotics have become ubiquitous in the environment due to their widespread use in human and veterinary medicine, as well as in agriculture. These compounds can persist in sediments for extended periods, potentially impacting microbial processes and ecosystem functioning. However, the effects of antibiotic pollutants on bacteria may be attenuated by natural minerals. The interaction between pollutants and minerals, such as manganese oxides, was investigated, with a focus on the potential for natural manganese oxides to mitigate the environmental impact of antibiotics in sedimentary systems. Therefore, my PhD thesis focuses on the effects of antibiotics on denitrification bacteria and processes in river sedimentary ecosystems, with a specific emphasis on the impact of antibiotic pollutants on microbial growth and denitrification activity.

In the first part of this PhD work, the antibiotic resistance in a non-pathogenic, denitrifying bacteria *Pseudomonas veronii* was investigated. *P. veronii* was tested towards a range of antibiotics via the classical disc method as well as a cultivation method allowing to determine minimal inhibiting concentrations (MICs). *P. veronii* was resistant towards macrolides (erythromycin and tylosin), tetracycline and the quinolone flumequine. On the other hand, *P. veronii* was susceptible towards the quinolones (ciprofloxacin and ofloxacin). In parallel antibiotic resistance genes in the genome of the denitrifier were identified via annotation through MaGe from the MicroScope platform. The genotypic antibiotic resistance of the denitrifier *P. veronii* showed the presence of several genes encoding efflux pumps (e.g. AcrAB-TolC). The presence of efflux pump genes most likely explains resistance towards the other antibiotics tested. The experimental results of *P. veronii* under denitrification condition demonstrate that different antibiotics have varying negative impacts on denitrification capabilities, with the nitrite reduction process being more susceptible to antibiotic impact than the nitrate reduction process.

In the second part, we first studied the mechanisms of transformation of ciprofloxacin (CIP) and tetracycline (TC) by a common redox soil mineral, birnessite (MnO<sub>2</sub>), and then the effects of transformation byproducts on both growth and activity on a denitrifying isolate. At pH 5, four different byproducts of CIP and five oxidation byproducts of TC were identified after reaction with birnessite identified by liquid chromatography-mass spectrometry, and monitored over a range of reaction time (0-6 hours). For CIP, impact on the growth of denitrifier *Pseudomonas veronii* decreased over reaction time where only the first CIP

byproduct (m/z 334) resulting from the opening of piperazine ring kept some of its antimicrobial activity. Closed batch experiments showed that the nitrite reduction rate was affected by CIP or byproducts while nitrate reduction rates remained stable. For TC, the antimicrobial activity of TC byproducts disappeared with the reaction processing, as well as the nitrate/nitrite reduction inhibition activity. The activities of nitrite reductases appeared to be affected by CIP and TC, compared to nitrate reductases.

The third part focused on the transport of ciprofloxacin (CIP) and tetracycline (TC) in sediments from the Seine Estuary in France, under nitrate reducing conditions. Dynamic flow experiments showed that although TC and CIP strongly interacted with the sediment components through adsorption and (bio)-chemical transformation, they kept their antimicrobial activities. Less nitrate reduction was observed during the first period of breakthrough, while TC and CIP were absent in the column effluent. Batch experiments with freeze-dried vs fresh sediments showed that adsorption and abiotic degradation are the major removal processes, while microbe-driven transformation is of less importance. Whereas TC is to a large extent chemically transformed and little adsorbed in the sediment, CIP was less transformed and more adsorbed, most likely due to the great reactivity of TC with redox-active mineral surfaces.

These findings improve our understanding of the antimicrobial activity and toxicity of antibiotics and their oxidation by-products, especially against environmental bacteria. By developing a better understanding of antibiotic-mineral-microbe interactions, we can improve our ability to predict and manage the cycling of nitrogen and other essential nutrients in the environment, which has important implications for sustainable agriculture, water quality management, and global biogeochemical cycles.

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# **Chapter 1**

## **Introduction**



# 1. Antibiotics

## 1.1. The widely use of antibiotics

Antibiotics are the products of rapid innovations in the health sector and their usage has changed the pattern of modern way of living. Ever since it has been recognized that they can be used as a medicine to treat and prevent infectious diseases, their market has been expanded. They have been extensively and effectively used in human and veterinary medicine and their benefits have also been recognized in agriculture, aquaculture, and livestock as growth promoters (Amarasiri et al. 2020, Baquero et al. 2008, Berendonk et al. 2015).

There are several different kinds of antibiotics and they can be classified based on their chemical structure, action mechanism, action spectrum, and the route of administration. Out of these classifications the most popular one is their mechanism of action and based on it the most common groups are:  $\beta$ -lactams, sulfonamides, aminoglycosides, glycopeptides, lincomycin, macrolides, rifamycin, tetracyclines, chloramphenicol and quinolones. Table 1-1 below contains further information.

Table 1-1 Antibiotics classification with their representative drugs, mode of action, and infection cured by them (Gothwal & Shashidhar 2015)

No.	Class of antibiotic	Representative drug	Mode of action
1	$\beta$ -Lactam	Phenoxyphenicillin, Oxacillin, Amoxicillin, Carbenicillin, Piperacillin	Inhibits steps in cell wall synthesis and murein assembly
2	Aminoglycosides	Gentamycin	Inhibits protein synthesis
3	Chloramphenicol	Chloramphenicol	Inhibits protein synthesis
4	Fluoroquinolones	Ciprofloxacin, Norfloxacin, Ofloxacin, Enrofloxacin, Enoxacin, Sarafloxacin	Inhibits DNA replication
5	Glycopeptides	Vancomycin	Inhibits steps in murein biosynthesis and assembly
6	Lincosamides	Clindamycin, Lincomycin	Inhibits protein synthesis
7	Macrolides	Clarithromycin, Erythromycin, Oleandomycin, Tylosin	Inhibits protein synthesis
8	Quinolones	Oxolinic acid, Nalidixic acid, Pipemidic acid, Flumequine, Pefloxacin	Inhibits DNA replication
9	Rifamycins	Rifamycin	Inhibits transcription (bacterial RNA polymerase)
10	Sulfonamides	Sulfamethoxazole, Sulfamethazine	Inhibitors of bacterial $\beta$ -lactamases
11	Tetracyclines	Tetracycline, Chlortetracycline, Oxytetracycline	Inhibits protein synthesis

Antibiotics are consumed from 100,000 tons to 200,000 tons per year all over the world, in both human and veterinary application (Wise 2002). It is estimated that 16,000 tons of antimicrobial agents are used annually in the United States, of which 70% are antibiotics (Sassman & Lee 2005a). The summary report on antimicrobials sold or distributed for use in food-producing animals, published by the U.S. food & drug administration (FDA), showed that 11,468 tons of antibiotics were actively marketed in 2019 in the U.S. The use of antibiotics is

increasing year by year, also in Europe. According to the annual report from the European Centre for Disease Prevention and Control (ECDC), in 2019, the average total clinical antibiotic consumption for systemic use in the European Union was 1.5 times higher than the world's average. Data from the French Institute for Public Health Surveillance in 2019 showed that, even though the number of antibiotic prescriptions in France decreased by 15% from 2009 to 2018, France still ranks third from the top in Europe in terms of clinical antibiotic usage.

The use of antibiotics added to feed for industrial livestock production has exploded since the 1980. From 1985 to 2001, antibiotic usage rose with 50% in the United States, and up to 80% of the total antibiotics used are given to farming animals. A report prepared by the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project (2011) showed that tetracycline, penicillin, and sulfonamide are the top three antimicrobial classes sold in tones, accounting for more than 80% of total sales. Data from the National agency for health security, food, environment and work ("Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail", ANSES, 2019) showed that, overall animal exposure against antibiotics has increased by 0.7% in 2018, especially for cattle and rabbits. It seems that the reduction in use is reaching a limit for certain families of antibiotics. The World Bank warns that, by 2050, drug-resistant infections could cause global economic damage on par with the 2008 financial crisis.

## 1.2. Antibiotics in the environment

Antibiotics are produced by bacteria, fungi and other microorganisms or synthesized by artificial chemistry, in order to fight against bacterial pathogens as they inhibit bacterial growth or kill bacteria.

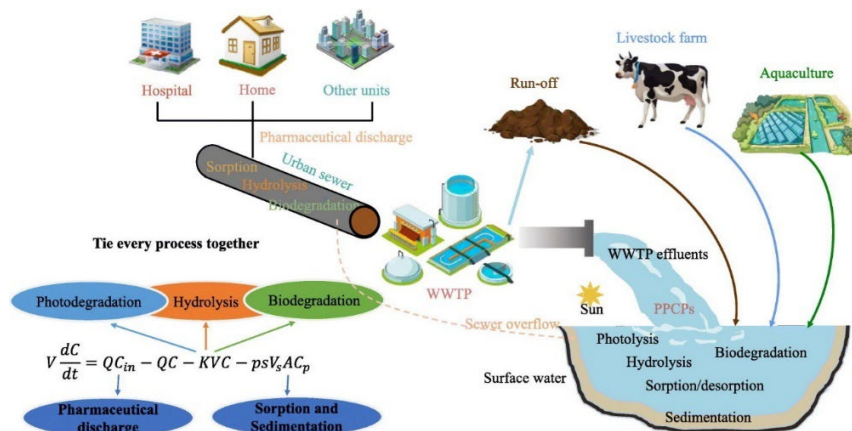


Figure 1-2 Fate and transport of antibiotics in water systems (Bavumiragira & Yin 2022)

Since their discovery, antibiotics have been widely used in the treatment and control of bacterial infectious diseases in humans and animals. However, antibiotics cannot be fully absorbed by the body; 40% - 90% of them are excreted (Halling-Sørensen et al. 1998, Jjemba 2002, Phillips et al. 2004). This part of antibiotics will enter the environment (Figure 1-2) along with sewage treatment plant, sludge or organic fertilizer application, as well as leakage, irrigation, fish pond bottom mud application and other artificial approach (Haller et al. 2002).

At present, antibiotics have been detected in solid matrices in worldwide, among which macrolides, quinolones, tetracyclines and sulfonamides are the main ones with high detection rate (Campagnolo et al. 2002, Kumar et al. 2005, Sassman & Lee 2005b). Some examples of solid matrix detected antibiotics and their concentrations are given in Table 1-3, indicating that the presence of antibiotics in the environment has become a worldwide problem.

Table 1-3 Concentrations of antibiotics in sludge, sediment, and soil

Antibiotic classes	Concentration $\mu\text{g kg}^{-1}$	Country	Year	Matrix	Reference
<b>Macrolides</b>					
<b>Erythromycin</b>	39	China	2012	Sludge	(Gao et al. 2012)
	111.1	Spain	2009	Sludge	(Radjenović et al. 2009)
	26.3–33.5	Spain	2011	Sediment	(da Silva et al. 2011)
	185	Italy	2010	Sludge	(Zuccato et al. 2010)
	10.4	China	2013	Sludge	(Li et al. 2013)
<b>Fluoroquinolones</b>					
<b>Norfloxacin</b>	3200	China	2012	Sludge	(Gao et al. 2012)
	76.5–1285.0	China	2013	Manure	(Huang et al. 2013)
	3.8–13.7	China	2013	Soil	(Huang et al. 2013)
	5399	China	2013	Sludge	(Li et al. 2013)
	6.6	Kenya	2020	Sediment	(Kairigo et al. 2020)
	1.6–225	France	2017	Sediment	(Dinh et al. 2017)
	271	Brazil	2019	Sludge	(Bisognin et al. 2021)
<b>Ciprofloxacin</b>	460	China	2012	Sludge	(Gao et al. 2012)
	5.8–17.1	China	2013	Soil	(Huang et al. 2013)
	20.5–520.0	China	2013	Manure	(Huang et al. 2013)
	285	China	2013	Sludge	(Li et al. 2013)
	29.3–47.4	Kenya	2020	Sediment	(Kairigo et al. 2020)
	2.9–569	France	2017	Sediment	(Dinh et al. 2017)
	2.09	Italy	2010	Sludge	(Zuccato et al. 2010)
	2217	Brazil	2019	Sludge	(Bisognin et al. 2021)
<b>Sulfonamides</b>					
<b>Sulfamethoxazole</b>	11	China	2012	Sludge	(Gao et al. 2012)
	0.2	China	2012	Sediment	(Chen & Zhou 2014)
	3.4–44.7	Kenya	2020	Sediment	(Kairigo et al. 2020)
	2.5–7.2	France	2017	Sediment	(Dinh et al. 2017)
	84.4	Spain	2013	Biosolids	(Pamreddy et al. 2013)
	4.7	Spain	2009	Sludge	(Radjenović et al. 2009)
	3.90	China	2013	Sludge	(Li et al. 2013)
<b>Tetracyclines</b>					
<b>Tetracycline</b>	22.3–117.0	China	2013	Manure	(Huang et al. 2013)
	100.5–600.0	China	2013	Sludge	(Huang et al. 2013)
	<2.6	China	2013	Soil	(Huang et al. 2013)
	2.96–72.9	China	2011	Sediment	(Yang et al. 2010)
	3.5	China	2014	Sediment	(Chen & Zhou 2014)
	62	Brazil	2019	Sludge	(Bisognin et al. 2021)

With the excessive use of antibiotics, a large number of antibiotics end up in aquatic sediments through various ways. Accumulation occurs due to the adsorption characteristics of the

sediments towards antibiotics (Kerrigan et al. 2018). Moreover, the continuous input of antibiotics leads to the “persistence” of antibiotics in sediments, which directly or indirectly affects environmental microorganisms, animals and plants (Hu et al. 2012), and induces the emergence of antibiotic resistant bacteria and emergence of resistance genes (Gothwal & Shashidhar 2015, Tian et al. 2012). Finally, antibiotics can enter the food chain through drinking water sources and plants and thus threaten human health.

### 1.2.1. Physicochemical properties of antibiotics

Antibiotics are ionic polar organic compounds. They generally contain multiple ionic functional groups and multiple acid dissociation constants. With the change of pH, they can perform a variety of valence states such as cations, neutral ions and anions, and generally have strong hydrophilicity. The main physical and chemical properties of several typical antibiotics are listed in Table 1-4.

Table 1-4 List of some antibiotics with important physicochemical properties

Antibiotic	Formula	MW	log $K_{ow}$	VP	WS	p $K_{a1}$	p $K_{a2}$	p $K_{a3}$	p $K_{a4}$
<b>Amoxicillin</b>	C <sub>16</sub> H <sub>19</sub> N <sub>3</sub> O <sub>5</sub> S	365.4	0.87	$2.53 \times 10^{-6}$	3433	2.69	7.49	9.63	—
<b>Ciprofloxacin</b>	C <sub>17</sub> H <sub>18</sub> FN <sub>3</sub> O <sub>3</sub>	331.34	0.28	$3.8 \times 10^{-11}$	<1000	3.32	5.59	6.18	8.76
<b>Norfloxacin</b>	C <sub>16</sub> H <sub>18</sub> FN <sub>3</sub> O <sub>3</sub>	319.33	-1.03	—	280	3.10	5.55	6.27	8.71
<b>Ofloxacin</b>	C <sub>18</sub> H <sub>20</sub> FN <sub>3</sub> O <sub>4</sub>	361.4	-0.39	$1.31 \times 10^{-10}$	28300	—	5.20	5.98	8.00
<b>Sulfadiazine</b>	C <sub>10</sub> H <sub>10</sub> N <sub>4</sub> O <sub>2</sub> S	250.3	-0.09	—	77	2.10	6.28	—	—
<b>Sulfamethoxazole</b>	C <sub>10</sub> H <sub>11</sub> N <sub>3</sub> O <sub>3</sub> S	253.3	0.89	$1.74 \times 10^{-5}$	610	1.83	5.57	—	—
<b>Tetracycline</b>	C <sub>22</sub> H <sub>24</sub> N <sub>2</sub> O <sub>8</sub>	444.4	-1.37	—	231	3.30	7.68	9.69	—
<b>Doxycycline</b>	C <sub>22</sub> H <sub>24</sub> N <sub>2</sub> O <sub>8</sub>	444.4	-0.02	—	50000	3.02	7.97	9.15	—
<b>Clarithromycin</b>	C <sub>38</sub> H <sub>69</sub> NO <sub>13</sub>	747.5	3.16	$3.1 \times 10^{-23}$	0.33	8.99	—	—	—
<b>Erythromycin</b>	C <sub>37</sub> H <sub>67</sub> NO <sub>13</sub>	733.5	3.06	$2.83 \times 10^{-23}$	2000	8.88	—	—	—

MW = molecular weight (g mol<sup>-1</sup>);  $K_{ow}$  = octanol–water partition coefficient; VP = vapor pressure (Pa);

WS = water solubility (mg L<sup>-1</sup>); p $K_a$  = dissociation constant.

The mother nuclei of four typical antibiotic families are shown in Figure 1-5. **Tetracycline** is the common name of among others tetracycline, oxytetracycline, and chlortetracycline. These antibiotics have the same basic mother nucleus (Figure 1-5a), hydrogenated paratetrene, which is a compound with different substituents. **Sulfonamides** contain a benzene ring, a para-amino group and a sulfonamide group (Figure 1-5b). A large number of effective derivatives, such as sulfadiazine and sulfamethazine, have been synthesized by substituting different groups for hydrogen atoms on the sulfonamide group. **Macrolide** antibiotics are based on macrolide moiety, which structure linked to 1-3 sugar molecules by a glycoside bond (Figure 1-5c). At least one sugar molecule is replaced by dimethylamine, tylosin, erythromycin and

spiramycin are representative molecules of macrolides. **Quinolones** can be divided into three generations. The third generation, i.e. fluoroquinolones, is the most widely used. Its structure contains adjacent carbonyl and carboxyl groups (Figure 1-5d), mainly including ciprofloxacin, ofloxacin, norfloxacin, etc.

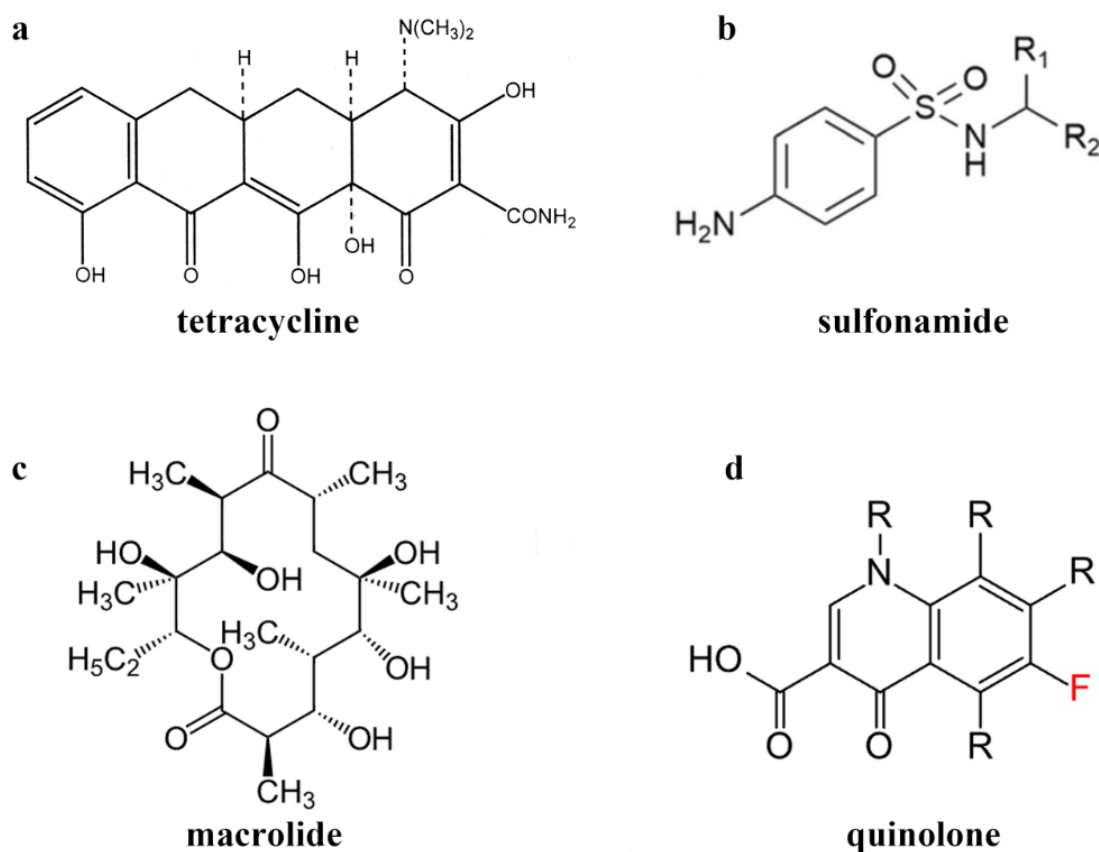


Figure 1-5 Mother nucleus of four typical antibiotic families.

### 1.2.2. Fate of antibiotics in the environment

In the solid phase, antibiotics are subject to a series of physicochemical and biological processes resumed in Figure 1-2 (page 4).

- Adsorption, migration, transformation

The physical sorption capacity of antibiotics on soil or sediments, expressed by the  $K_d$  value, varies from low to high sorption, i.e. from 0 to 6,000 L kg<sup>-1</sup> corresponding to different molecules under different conditions (Tolls 2001). The sorption depends on several environmental factors such as organic matter, pH and clay type (Conde-Cid et al. 2020, Tolls 2001) and the different chemical structures of antibiotic molecules (Ozumchelouei et al. 2020).

In sediments, the persistence of antibiotics varies greatly, from less than one day to weeks or even months. Generally speaking, the physical and chemical properties of antibiotics, like the octanol/water partition coefficient ( $K_{ow}$ ), acid dissociation constant ( $K_a$ ) and polarity, indicate their retention tendency in solid-phase.

Table 1-6 gives an overview of reported adsorption coefficient values of some representative antibiotics that are widely used / studied, showing the range in  $K_d$  values.

Table 1-6 Reported adsorption coefficient values of antibiotics

Antibiotic	Matrices	pH	OC (%)	$K_d$ (L kg <sup>-1</sup> )	Reference
<b>Macrolides</b>					
<b>Erythromycin</b>	Clay loam	6.45	0.8	130	(Pan & Chu 2016)
	Marine sediments	—	—	337	(Xu et al. 2009)
<b>Fluoroquinolones</b>					
<b>Ciprofloxacin</b>	Loamy sand	5.30	0.7	427	(Nowara et al. 1997)
	Loamy sand	6.9	10	934–4844	(Conkle et al. 2010)
	Soil, clay, and sand fraction	4.9–8.7	0.3–8.9	74–54600	(Figueroa-Diva et al. 2010)
<b>Norfloxacin</b>	Clay loam	6.45	0.8	591	(Pan & Chu 2016)
	Loamy sand	6.9	10	1942–5791	(Conkle et al. 2010)
	Soil, clay, and sand fraction	4.9–8.5	0.3–8.9	41–36400	(Figueroa-Diva et al. 2010)
<b>Tetracyclines</b>					
<b>Tetracycline</b>	Clay loam	6.45	0.8	1093	(Pan & Chu 2016)
	Biosolids	6.9	—	7315	(Wu et al. 2009)
	Soil, clay, and sand fraction	5–8.6	0.3–8.9	41–8300	(Figueroa-Diva et al. 2010)
<b>Sulfonamides</b>					
<b>Sulfamethazine</b>	Clay loam	6.45	0.8	1.37	(Pan & Chu 2016)
	Sandy loam soil/sandy	5.5–9	—	0.23–0.58	(Lertpaitoonpan et al. 2009)
	Biosolids	—	—	7.5	(Wu et al. 2009)
	Sandy loam and loam	5.0–9.0	—	0.9–18	(Wegst-Uhrich et al. 2014)

Oxytetracycline, tetracycline and sulfonamides are mostly weak acid or weak base compounds, with strong affinity for sediments, it is proved that soil adsorption is a significant factor to reduce the concentration of antibiotics in water phase (Ying et al. 2002). Rabølle et al (2000) studied the adsorption and movement of four different antibiotics in different solid-phases. Among them, the distribution coefficient ( $K_d$ ) of metronidazole was determined being 0.5–0.7 L kg<sup>-1</sup>, olaquinox was 0.7–1.7 L kg<sup>-1</sup>, tylosin was 8–128 L kg<sup>-1</sup>. Meanwhile oxytetracycline had a high adsorption capacity in the investigated solid-phase, and no obvious desorption was observed. Its  $K_d$  value was determined being 417–1026 L kg<sup>-1</sup>, which variousity may be due to the formation of a complex between oxytetracycline and divalent ions (Lunestad & Goksøyr 1990). Boxall et al found that the  $K_d$  of sulfachlorpyridazine in soil was considerably low (about 0.9–18.1 L kg<sup>-1</sup>), which indicated that it had strong migration ability (Boxall et al. 2002). The following field experiments also confirmed the idea that sulfachlorpyridazine migrated to the surface water rapidly after being applied to the soil, and its detected concentration in the drainage system was up to 590 µg L<sup>-1</sup>. However, the retention time of some antibiotics in the sediment under laboratory conditions (Bakal & Stoskopf 2001) was longer than under nature

environment (Galloway et al. 2004). In fact, this may be due to factors such as matrix differences of tested soil or sediment. According to Aboul-kassim et al, many factors could affect the adsorption of antibiotics in sediments, such as pH, cation exchange capacity (CEC), organic matter;  $K_d$  values can be considered as the result of interaction between many factors. This is actually similar to other organic pollutants (Jia et al. 2005).

#### - Degradation

Chemical and biological modifications are mainly degradation, including both abiotic (oxidation, reduction, hydrolysis and photodegradation) as well as biotic processes (biodegradation) (Conde-Cid et al. 2020). The magnitude of antibiotic degradation depends on environmental factors (such as pH, organic carbon content, bacteria population, etc.) and antibiotic properties (solubility, stability, etc.) (Boxall 2008, Sarmah et al. 2006). Thus, the degradation rates for different antibiotics are show large variation, with half-lives ranging from a few days to several months (Boxall 2008).

**Hydrolysis** is generally considered one of the most important pathways for abiotic degradation of antibiotics.  $\beta$ -lactams are especially susceptible to hydrolytic degradation, whereas macrolides and sulfonamides are known to be less susceptible to hydrolysis (Braschi et al. 2013, Mitchell et al. 2015). **Photo-degradation**, which contributes to degradation of antibiotics (e.g., quinolones and tetracyclines) spread on the soil surface during application of manure and slurry to agricultural land, is another important abiotic degradation process (Thiele-Brun & Peters 2007).

In addition to abiotic processes, **microbial degradation** may contribute to disappearance of antibiotics in soil. Some bacteria that degrade antibiotics by their oxidase/reductase have been isolated from antibiotic-contaminated soils. For example, strains belonging to the genera *Microbacterium* (Topp et al. 2016), *Burkholderia* (Zhang & Dick 2014), *Stenotrophomonas* (Leng et al. 2016), *Labrys* (Mulla et al. 2018), *Ochrobactrum* (Zhang et al. 2017), and *Escherichia* (Mulla et al. 2018, Wen et al. 2018) were capable of degrading sulfamethazine, penicillin G, tetracycline, erythromycin and doxycycline in liquid cultures, respectively. Other bacteria belonging to the genera *Klebsiella* (Xin et al. 2012), *Acinetobacter*, *Escherichia* (Zhang et al. 2012), *Microbacterium* (Kim et al. 2011), *Labrys* (Amorim et al. 2014), and *Bacillus* (Rafii et al. 2009) that were capable of degrading chloramphenicol, sulfapyridine, sulfamethazine, ciprofloxacin, norfloxacin, and ceftiofur have been isolated from patients, sediments, sludge,

animal feces, and seawater.

Different degradation processes typically occur simultaneously under environmental conditions. The effect of environmental conditions on degradation of antibiotics was demonstrated for oxytetracycline in the sediment of a fishpond (Jacobsen & Berglind 1988). The oxytetracycline could maintain its antibacterial activity within 12 weeks in the sediment under oxic conditions (10 mg L<sup>-1</sup> oxygen), while in the anoxic sediment, oxytetracycline retention was relatively strong – with a half-life time up to 10 weeks. Others such as chloramphenicol can be degraded by nitrated sludge, indicating that the presence of nitrate may affect the degradation process (Campos et al. 2001). It is also reported that some sulfonamides were confirmed to have the same degradability in activated sludge (Ingerslev & Halling - Sørensen 2000, Richardson & Bowron 1985). Warman et al (1981) detected tetracycline residues in the soil where poultry manure was applied, proving that the antibiotic metabolites (such as glucosidic acid) discharged by the animals receiving the medicine were degraded by microbial activities in the liquid manure, and converted into active drugs again. Amoxicillin is relatively stable in the feces, but it is obviously easy to degrade in the environment (Christian et al. 2003). Walters et al (Walters et al. 2010) conducted an outdoor mesocosm study to explore the degradation of several compounds in soils amended with biosolids over a 3-year period. Various antibiotics, including ofloxacin, ciprofloxacin, norfloxacin, doxycycline, and tetracycline, had varying degrees of persistence, with half-lives of 1,386, 2,310, 1,155, 533, and 578 days, respectively. This shows that antibiotics can persist for years in the soil environment under ambient conditions and therefore should be investigated further.

### **1.2.3. The impact of antibiotics on environmental microbially relevant processes**

In recent years, antibiotics, as an emerging pollutant, have been widely studied. Researchers from different countries have carried out studies on its type, distribution, content, and migration and transformation process in the sediment environment (Table 1-3).

The deleterious effect of antibiotics on natural microbial communities could be direct or indirect. The direct action could be that some microbial groups involved in important ecosystem functions disappear or are inhibited by bactericidal and bacteriostatic effects. However, as the indirect action, antibiotics may act as a selective force on some microbial populations that can develop resistance, leading to genetic and phenotypic variability and

affecting various physiological activities; in some cases, bacteria may develop degradation ability as a homeostatic response.

Antibiotics can cause changes in the structure of natural bacterial communities (disappearance or inhibition of certain bacterial groups) in the environment (Allen et al. 2010). The effects can be detected even in non-target organisms with important ecological functions (Pallecchi et al. 2008, Woegerbauer et al. 2015). For example, sulfonamides have been found to cause a change in microbial diversity by reducing not only microbial biomass but also the ratio of bacteria to fungi (Underwood et al. 2011).

The impact of antibiotics on bacterial communities has resulted in a significant influence on biochemical processes, particularly in the nitrogen cycle, within natural environments. It has been reported that nitrification and anammox activities could be inhibited at therapeutic doses, while environmentally relevant concentrations of antibiotics like sulfonamides and fluoroquinolones could partially inhibit denitrification (Roose-Amsaleg & Laverman 2016). Denitrification is one of the representative environmental microbially relevant processes. The effects of different antibiotics on denitrification have been investigated, and the results vary considerably among the different solid matrices and concentrations tested (Table 1-7, next page). Antibiotics including amoxicillin, erythromycin, tylosin, bacitracin, clarithromycin, difloxacin, florfenicol, chloramphenicol, thiamphenicol, ciprofloxacin, flumequine, ofloxacin, tetracycline, chlortetracycline, oxytetracycline, roxarsone, triclosan, triclocarban, narasin, gentamicin, lincomycin, sulfamethazine, sulfamethoxazole and sulfadiazine were reported to have negative effect on denitrification in soil, sediment, sludge, landfill or groundwater. The effective concentrations of these antibiotics were ranging from several ppt to thousands ppm, combined with a wide range of exposure times. Interestingly, denitrification inhibition caused by some antibiotics were found to be reversible under low exposure concentration around several ppb to ppm.

Table 1-7. Overview of the studies investigating the effects of antibiotics on the denitrification process

Antibiotic	Concentration range	Matrix type	Exposure time	Effects on denitrification	Reference
amoxicillin	ppm	sediment	hours	inhibition (37%)	Costanzo et al. 2005
Bacitracin	ppm	soil	days	inhibition	Banerjee et al. 2013
chloramphenicol	ppm	soil	hours	inhibition (19-41%)	Murray et al. 1999
chlortetracycline	ppb	groundwater	days	inhibition (15-31%)	Ahmad et al. 2014
ciprofloxacin	ppb	soil	days	no effect	Conkle and White, 2012
ciprofloxacin	ppb	sediment	hours	no effect	Costanzo et al. 2005
ciprofloxacin	ppm	enrichment cultures	weeks	inhibition (25%)	Liu et al. 2014
clarithromycin	ppm	sediment	hours	inhibition (~ 37%)	Costanzo et al. 2005
difloxacin	ppm	soil	weeks	inhibition (50%)	Kotzerke et al. 2011
erythromycin	ppm	sediment	hours	inhibition (~ 37%)	Costanzo et al. 2005
florfenicol	ppb	soil	month	inhibition	Wang et al. 2020
flumequine	ppb	sediment	weeks	inhibition (41 %)	Yan et al. 2013
gentamicin	ppb	soil	days	reversible inhibition	DeVries et al. 2015
lincomycin	ppb	soil	days	reversible inhibition	D'alessio et al. 2019
monensin	ppb	soil	days	no effect	D'alessio et al. 2019
narasin	ppt	soil	days	reversible inhibition	DeVries et al. 2015
ofloxacin	ppb	wetland	month	inhibition	Tong et al. 2019
oxytetracycline	ppm	landfill	hours	increasing N <sub>2</sub> O	Wu et al. 2017
oxytetracycline	ppm	landfill	month	increasing N <sub>2</sub>	Wu et al. 2017
oxytetracycline	ppb	sediment	month	inhibition	Zou et al. 2018
roxarsone	ppm	soil	days	inhibition	Banerjee et al. 2013
sulfadiazine	ppt	soil	days	reversible inhibition (34%)	DeVries et al. 2015
sulfadiazine	ppm	soil	weeks	inhibition (~ 70%)	Kotzerke et al. 2008
sulfadiazine	ppm	soil + manure	weeks	inhibition (28%)	Radl et al. 2015
sulfadimidine	ppb	sediment	month	inhibition (21-26%)	Wang et al. 2019
sulfamethazole	ppb	groundwater	days	Inhibition (17-82%)	Ahmad et al. 2014
sulfamethazine	ppb	soil	days	reversible inhibition	D'alessio et al. 2019
sulfamethazine	ppb	sediment	hours	inhibition (20-30%)	Hou et al. 2015
sulfamethazine	ppb	soil	hours	inhibition (14–24%)	Shan et al. 2018
sulfamethazine	ppm	landfill	hours	inhibition (98%)	Wu et al. 2017
sulfamethazine	ppb	landfill	month	inhibition (98%)	Wu et al. 2017
sulfamethazine	ppb	soil	days	inhibition	D'Alessio et al. 2019
sulfamethoxazole	ppm	anoxic sludge	days	inhibition	An and Qin. 2018
sulfamethoxazole	ppb	soil	days	inhibition	Conkle and White. 2012
sulfamethoxazole	ppt	soil	days	inhibition	DeVries et al. 2015
sulfamethoxazole	ppb	manured soil	month	no effect	Rahman et al. 2018
sulfamethoxazole	ppb	groundwater	weeks	inhibition (47%)	Underwood et al. 2011
sulfamethoxazole	ppm	sediment	weeks	inhibition (39%)	Yan et al. 2013
tetracycline	ppm	soil	hours	inhibition (37%)	Chen et al. 2019
tetracycline	ppb	soil	days	no effect	Conkle and White. 2012
tetracycline	ppm	sediment	weeks	no effect	Roose-Amsaleg et al. 2013
tetracycline	ppm	soil	days	inhibition (82%)	Semedo et al. 2018
tetracycline	ppm	soil	hours	inhibition (23–72%)	Shan et al. 2018
tetracycline	ppm	sediment	hours	inhibition	Zhang et al. 2019
thiamphenicol	ppb	sediment	weeks	inhibition	Yin et al. 2016
triclocarban	ppm	soil	hours	inhibition (30%)	Chen et al. 2019
triclosan	ppm	soil	hours	inhibition (27%)	Chen et al. 2019
tylosin	ppm	anaerobic digesters	month	inhibition	Zhang et al. 2019
vancomycin	ppb	sediment	weeks	no effect	Yan et al. 2013
Virginiamycin	ppm	soil	days	no effect	Banerjee et al. 2013

## 2. Nitrogen in the environment

Nitrogen cycling is of paramount importance as it plays a critical role in maintaining the balance of life on Earth. Microbial processes dominate this cycle, making it an essential element for the functioning of ecosystems. However, the delicate balance of the nitrogen cycle can be disrupted by a variety of environmental and anthropogenic factors. Understanding the microbial processes in the nitrogen cycle and their impacting factors is crucial to ensure the long-term sustainability of our planet.

### 2.1. The Biogeochemical Nitrogen Cycle

#### 2.1.1. The global nitrogen cycle

The nitrogen cycle refers to the cyclic transformation process of nitrogen in the nature. It is one of the basic elemental cycles in the biosphere (Galloway et al. 2008). For example, dinitrogen in the atmosphere enters the terrestrial or aquatic compartment through the action of microorganisms (nitrogen fixation), and is used by animals and plants. In the atmosphere, this cycle repeats itself to infinity. A scheme to describe nitrogen cycle is shown below as Figure 1-8.

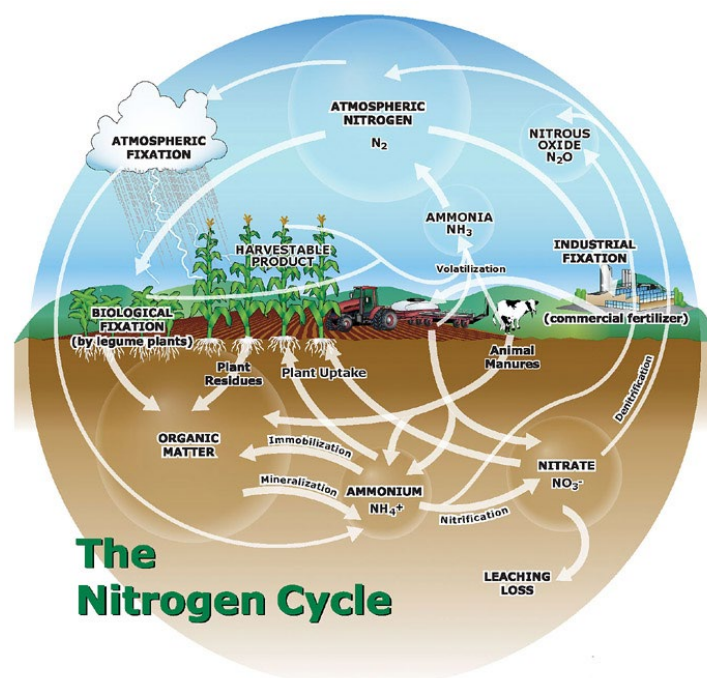


Figure 1-8 the global nitrogen cycle with the different sources (Source: *Nitrogen Notes*, published by International Plant Nutrition Institute, Canada)

### 2.1.2. The microbial N-cycle

The main processes of the nitrogen cycle in terrestrial ecosystems are (see Figure 1-9) (Stein & Klotz 2016): ammonification, including nitrogen fixation, and assimilatory and dissimilatory reduction of nitrite (reactions 1 and 2), nitrification (reactions 3A, 3B and 4), denitrification, including canonical, methane-oxidation-dependent denitrification and nitrifier-dependent (reactions 6A–D), anammox, as a form of coupled nitrification–denitrification (reactions 7A–C); and nitrite–nitrate interconversion (reactions 4 and 5). The general processes of organic matter mineralization and assimilation by cellular life complete the movement of reactive nitrogen through the biosphere.

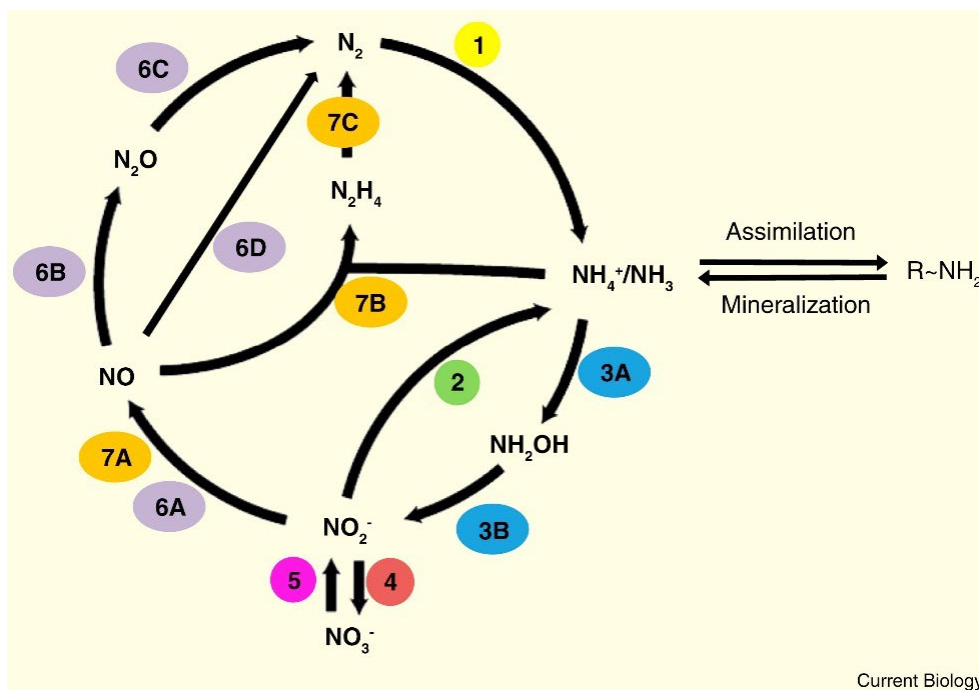


Figure 1-9: Major transformations in the nitrogen cycle (Stein & Klotz 2016). Reactions that comprise the seven major processes of the nitrogen cycle are represented by the numbered circles. Ammonification may be accomplished either by process 1, reduction of dinitrogen (also referred to as ‘nitrogen fixation’ or ‘Nif’), or by process 2, dissimilatory nitrite reduction to ammonium (DNRA). Nitrification is composed of process 3, oxidation of ammonia to nitrite (also referred to as ‘nitritation’), and process 4, oxidation of nitrite to nitrate (also referred to as ‘nitrataion’). Process 5, reduction of nitrate to nitrite, can be coupled to processes 2, 6 or 7 in a population or a community. Denitrification is shown as process 6, which is also referred to as ‘nitrogen-oxide gasification’. Anammox is shown as process 7, and is also referred to as coupled ‘nitrification–denitrification’.

The transformation of inert dinitrogen gas into bioavailable nitrogen starts with nitrogen fixation, which molecular nitrogen is reduced to ammonia and other nitrogen-containing compounds. There are two ways to fix dinitrogen ( $N_2$ ) in nature. One is non-biological nitrogen

fixation, that is, nitrogen fixation through lightning, high temperature discharge, etc., which forms limited numbers of nitrides; the other is biological nitrogen fixation, that is, the reduction of molecular nitrogen to ammonia via microbial activity. More than 90% of the molecular nitrogen in the atmosphere is reduced by the action of nitrogen-fixing microorganisms, due to their nitrogenase enzyme (Dixon & Kahn 2004, Howard & Rees 1996) that promotes the hydrogenation of nitrogen to ammonia.

Two types of microbial dinitrogen fixation exist: in the aquatic environment by cyanobacteria or in the terrestrial environment, in symbiosis with leguminous plants (such as peas or broad beans) (Howard & Rees 1996, Peoples et al. 1995, Vitousek et al. 2002). These interactions with plants allow microbial take use of the ammonium and nitrates in the soil, and assimilation of these inorganic nitrogens into organic nitrogen such as proteins in the plant. Animals use plants as food directly or indirectly, assimilating organic nitrogen in plants into organic nitrogen in animals. Organic nitrogen in organisms is synthesized via dinitrogen fixation.

The organic matter mineralization process consists of the decomposition by microorganisms of organic nitrogen compounds in the remains, effluents, and residues of animals and plants to form ammonia. Part of the ammonia produced is retained by microorganisms and plants, or fixed by clay minerals

Ammonia is used to produce ammonium ions ( $\text{NH}_4^+$ ). In oxygen-rich environments, these ions can be converted to nitrite ions ( $\text{NO}_2^-$ ) by ammonia oxidizing bacteria, and then converted to nitrate ions ( $\text{NO}_3^-$ ) by nitrite oxidizing bacteria. This process is called nitrification.

Inorganic nitrogen produced by ammonification and nitrification can be absorbed and utilized by plants. Under conditions of insufficient oxygen, the nitrate in the soil is reduced to nitrite by various microorganisms such as denitrifying bacteria, nitrogen is further reduced, and at last the molecular nitrogen is returned to the atmosphere. This process is called denitrification (Kuenen & Robertson 1994, Prosser 1990, Satoh et al. 2003, Sharma & Ahlert 1977, Verstraete & Focht 1977, Yoshioka & Saijo 1984).

### **2.1.3. Environmental implication of N cycle**

Nitrogen is an essential element for living creatures, but gaseous dinitrogen cannot be used directly by most of the plants and animals. Therefore, manufacture, or fixation, is an inevitable process for converting gaseous free dinitrogen into combined nitrogen that can be absorbed

by creatures. Dinitrogen conversion build a mutually beneficial symbiotic relationship, producing ammonia for plants in exchange for carbon, therefore make nitrogen-poor land fertile. It can be seen that the soil has become the most active area in the nitrogen cycle due to dinitrogen conversion activity, among which denitrifiers had a 90% contribution (Galloway et al. 1995, Giller 2001, Mylona et al. 1995, Peoples et al. 1995).

Nitrogen exists in various forms; its conversion and utilization are complex. After entering soil or sediment, ammonium ions are easily fixed in to the soil matrix (especially humus and clay), and undergo various transformation processes driven by microorganisms to form a variety of nitrogen species. Nitrate and nitrite ions are more mobile in the soil consist of positive ions (mainly humus) than that of negative ions, due to their negative electrical properties. Loss to groundwater of soluble nitrate and nitrite often occurs after rain or irrigation. Nitrogen thus migrates to ground and river water increase nitrogen concentrations, leading to environmental problems. Excessive nitrogen oxides cause nitrogen pollution in the water body, which not only endangers human health, but also becomes a factor of eutrophication of the water body. Nitrous oxide ( $N_2O$ ) is also an important greenhouse gas, which enhances the greenhouse effect after entering the atmosphere (Camargo &Alonso 2006, Castillo et al. 2000, Heaton 1986, Howarth 2008).

#### **2.1.4. Nitrogen imbalance**

Since the 1970s, humans have organized extensive research on the nitrogen cycle in ecosystems. SCOPE (Scientific Committee on Problems of the Environment) raised global nitrogen overload as a potential environmental issue and chemical time bomb. The increase in the amount of activated nitrogen has increased agricultural productivity, but it also brought pressure on the global ecological environment, and further cause ecological and environmental problems related to the nitrogen cycle, such as the greenhouse effect, water pollution and acid rain. Since 2006, the use of nitrogen fertilizers in the United Kingdom and the United States had been more severely restricted. Same restrictions took effect since 2015 in China. As the No. 1 country in European agricultural exports, nitrogen fertilizers usage in France is second only to Germany. Meanwhile, due to the extensive cultivation of leguminous plants (especially soybeans and alfalfa), the production of chemical fertilizers in human society, and the nitrogen-containing pollution components released by vehicles and thermal power stations, humans make far more nitrogen in bioavailable forms than before every year.

Excessive "activation" of nitrogen caused certain unbalance, and the nitrogen cycle is seriously disrupted.

The main environmental issues related to elevated nitrogen levels are:

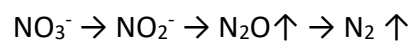
- The microbial conversion of ammonia to nitrates and acidic hydrogen ions, causes **acidification** of soil and water ecosystems and reducing biodiversity. In addition, ammonia is highly toxic to fish and other marine life.
- There had been a 30-fold increase in mean lake dissolved inorganic nitrogen (DIN) concentrations from 20 to 650  $\mu\text{g L}^{-1}$  in Europe and north American (Ann-Kristin et al. 2006). Too much nitrogen in water leads to **eutrophication**. Firstly, eutrophication of water bodies will cause the destruction of water resources, reducing the value of water usage, directly affecting human health, and at the same time increasing the cost of water treatment; secondly, nitrate-rich groundwater will cause a sharp increase of cyanobacteria and other algae, and triggers "algal bloom" and "red tide" and other phenomena, resulting in a large number of aquatic organisms dying due to hypoxia.
- Nitrous oxide ( $\text{N}_2\text{O}$ ) has a particularly strong ability to absorb infrared radiation, which acts 200 times higher than that of carbon dioxide, and thus causes the **greenhouse effect**. In addition to the greenhouse effect, nitrous oxide (commonly known as laughing gas) can also chemically react with ozone in the atmosphere, disturb the ozone layer, increase the intensity of ultraviolet light on the ground, and endanger human health. Nitric oxide and nitrogen dioxide are also one of the components of **acid rain**.
- $\text{NO}_2^-$  induces various diseases and even cancer. Once people get excessive amounts of nitrate from contaminated fruits, vegetables or water, high blood pressure, congenital central nervous system disability, and non-Hodgkin's lymphoma can occur. Nitrogen oxides, which produced by burning fossil fuels, can form ground-level ozone and trigger asthma. A large number of medical research have proved that the incidence of liver cancer (Grosse et al. 2006, Yang et al. 2007), stomach cancer (Hill et al. , Kim et al. 2008) and other symptoms (Bartsch &Montesano 1984) is also closely related to the amount of nitrate intake by the human body. The increase of nitrate content in groundwater is related to the safety of drinking water, because excessive nitrite ions in the water can affect the oxygen concentration in the blood of infants and children and cause methemoglobinemia or Blue-baby Syndrome (Gerardi 2003).

→ Municipalities must face social problems such as excessive  $\text{NO}_3^-$  in groundwater and drinking water and increased medical costs. In rural areas near estuary, excessive  $\text{NO}_3^-$  content in drinking water wells or farmland is also a problem.

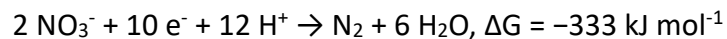
## 2.2. Denitrification

Denitrification refers to the biochemical process by which microbes (bacteria archaea and fungus mostly) reduce nitrate ( $\text{NO}_3^-$ ) to dinitrogen gas ( $\text{N}_2$ ) through a series of intermediate products ( $\text{NO}_2^-$ ,  $\text{NO}$ ,  $\text{N}_2\text{O}$ ). The microbes, mainly prokaryotes, involved in this process are called denitrifiers.

Denitrifying microbes use nitrate ( $\text{NO}_3^-$ ) as an electron acceptor to perform respiration under anaerobic conditions to obtain energy. They use  $\text{NO}_2^-$  and  $\text{NO}_3^-$  as the ultimate electron acceptors for respiration, reducing nitric acid to dinitrogen ( $\text{N}_2$ ), which is called denitrification:

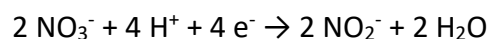


Many bacteria can perform denitrification, the physiological group is called denitrifying bacteria (Gamble et al. 1977). The total denitrification process and the Gibbs free energy can be expressed by the following formula (Mariotti et al. 1988, Payne 1981, Zilu 2011):

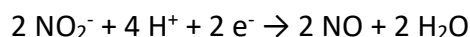


These include the following four reduction reactions:

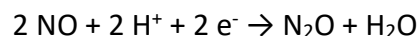
Reduction of nitrate ( $\text{NO}_3^-$ ) to nitrite ( $\text{NO}_2^-$ ):



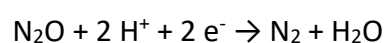
Reduction of nitrite ( $\text{NO}_2^-$ ) to nitric oxide ( $\text{NO}$ ):



Nitric oxide ( $\text{NO}$ ) reduction to nitrous oxide ( $\text{N}_2\text{O}$ ):



Reduction of nitrous oxide ( $\text{N}_2\text{O}$ ) to nitrogen ( $\text{N}_2$ ):



The enzymes that catalyze the four steps are nitrate reductase (*nar*), nitrite reductase (*nirK*)

and *nirS*), nitric oxide reductase (*nor*), and nitrous oxide reductase (*nosZ*) (Philippot 2002).

The above four reactions are all exothermic, so under anaerobic or hypoxic conditions, bacteria can use nitrate ( $\text{NO}_3^-$ ) as the terminal electron acceptor of the electron transfer chain to complete material energy exchange.

### 2.2.1. Denitrifiers – the executors of denitrification

Denitrifying bacteria exist widely in various forms in nature (Castignetti & Hollocher 1984, Heylen et al. 2006, Kartal et al. 2007, Lone et al. 2010, Patureau et al. 2000, Verstraete & Focht 1977). The taxonomic units are mainly distributed in families such as *Pseudomonaceae*, *Neisseriaceae*, *Nitrobacteraceae*, *Rhodospirillaceae*, *Bacillaceae*, *Cytophagaceae*, *Spirilaceae*, *Rhizobiaceae*, *Halobacteriaceae*.

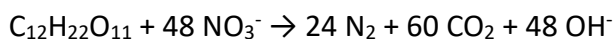
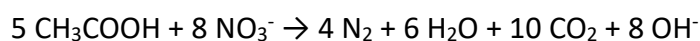
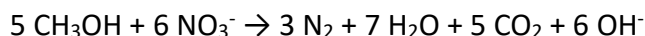
The traditional theory was that denitrifiers are bacteria and carry out denitrification under anaerobic conditions. A fungi named *Fusarium oxysporum* was found to have ability to reduce nitrate and nitrite to  $\text{N}_2\text{O}$  under anaerobic conditions, thus breaking this traditional concept (Shoun & Tanimoto 1991). Later, it was found that the denitrification process widely exists in fungi of the genus *Mycetes*, *Ascomycetes*, and *Basidiomycetes*; many actinomycetes (Kumon et al. 2002) include *Streptomyces* and *Frankia* (Dominique et al. 2000), and even some yeasts have denitrification capacity. Another finding is that denitrification also exists under aerobic conditions (Verstraete & Focht 1977), such as *Alcaligenes faecalis* and *Paracoccus denitrificans* can simultaneously use oxygen and nitrate as metabolism electron acceptors. The definition of denitrification is: a process in which microorganisms use nitrogen oxides as electron acceptors and transform it in gaseous nitrogen form to generate energy. The process consists of a four-step reaction to reduce nitrate to molecular nitrogen. Examples of well-studied denitrifiers are in table 1-10, their nutrition either heterotrophic or autotrophic is given.

Table 1-10 Examples of well-studied denitrifiers

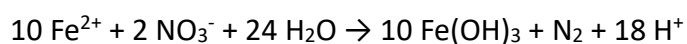
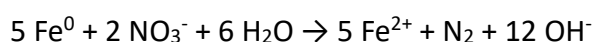
Species	Nutrition	Energy sources
<i>Pseudomonas stutzeri</i>	heterotrophic	organic carbon
<i>Paracoccus denitrificans</i>	autotrophic	hydrogen ( $\text{H}_2$ )
<i>Thiobacillus denitrificans</i>	autotrophic	sulfide (S) or thiosulfate ( $\text{S}_2\text{O}_3$ )

Most denitrifying bacteria are heterotrophic bacteria, such as *Micrococcus denitrificans*, *Pseudomonas aeruginosa*, *Ps. Atutzeri*, *Bacillus licheniformi*, etc. It means they use organic matter as a nitrogen source and energy source for anaerobic respiration. The reaction

equations of organic substrates, such as methanol, acetic acid and sucrose, are provided below:



A few denitrifying bacteria are autotrophic bacteria, such as *Thiobacillus denitrificans*, which oxidizes sulfur or nitrate to obtain energy, assimilate carbon dioxide, and uses nitrate as the terminal electron acceptor for respiration. The reaction equations of inorganic substrates, such as iron, are provided below:



### 2.2.2. Denitrifying microorganisms

More than 60 genera of denitrifying microorganisms have been identified including archaea and fungi (Philippot et al. 2007). Denitrifiers are widely distributed in natural environments such as soil, silt, and water. Microbes are considered to be the most important elements of the Earth's biosphere and play a key role in the material cycle. In the past 15 - 20 years, microbial ecology has undergone a revolutionary development. Due to the achievements of microbial molecular biology, genes and sequences of uncultured microorganisms can directly be obtained from nature without the need to isolate and culture the microorganisms. Among the methods studying microbes without cultivation, molecular biology using nucleic acids were developed. Concerning denitrifying organisms, gene encoding enzymes involved in the four step reactions (introduced in section 2.2) are targeted using the polymerase chain reaction Polymerase chain reaction (PCR) method.

PCR is a relatively simple and widely used molecular biology technique to amplify and detect DNA and RNA sequences. Standard PCR is widely used to amplify DNA for subsequent experimental use. Quantitative polymerase chain reaction (qPCR) is very similar to PCR, but it also allows to quantify the amount of target DNA present in a sample. As in standard PCR, DNA is amplified by 3 repeating steps: denaturation, annealing and elongation. However, in qPCR, fluorescent labeling enables the collection of data as PCR progresses. Henry et al developed a method for analysis of denitrifying bacteria in soil using real-time qPCR technology with *nirK*

as the target gene (Henry et al. 2004). This method can linearly determine  $10^7$  *nirK* gene copies per gram soil (dry weight) with a sensitivity of  $10^2$ . Gruntzig et al used *nirS* gene from *Pseudomonas stutzeri* as the target to design primers, and used real-time qPCR technology to quantitatively analyze denitrifying bacteria in environmental samples. The results showed that the quantitative determination of the target genes in the range of 1 to  $10^6$  copies (per gram soil [dry weight]) was linear relationship ( $r^2=0.999$ ) (Gruntzig et al. 2001). Today, qPCR (Figure 1-11) is the main method (Philippot et al. 2007), combined with metabarcoding and metagenomic methods (Francioli et al. 2021, Schmieder & Edwards 2012), for the quantification of denitrifiers used in soil environments.

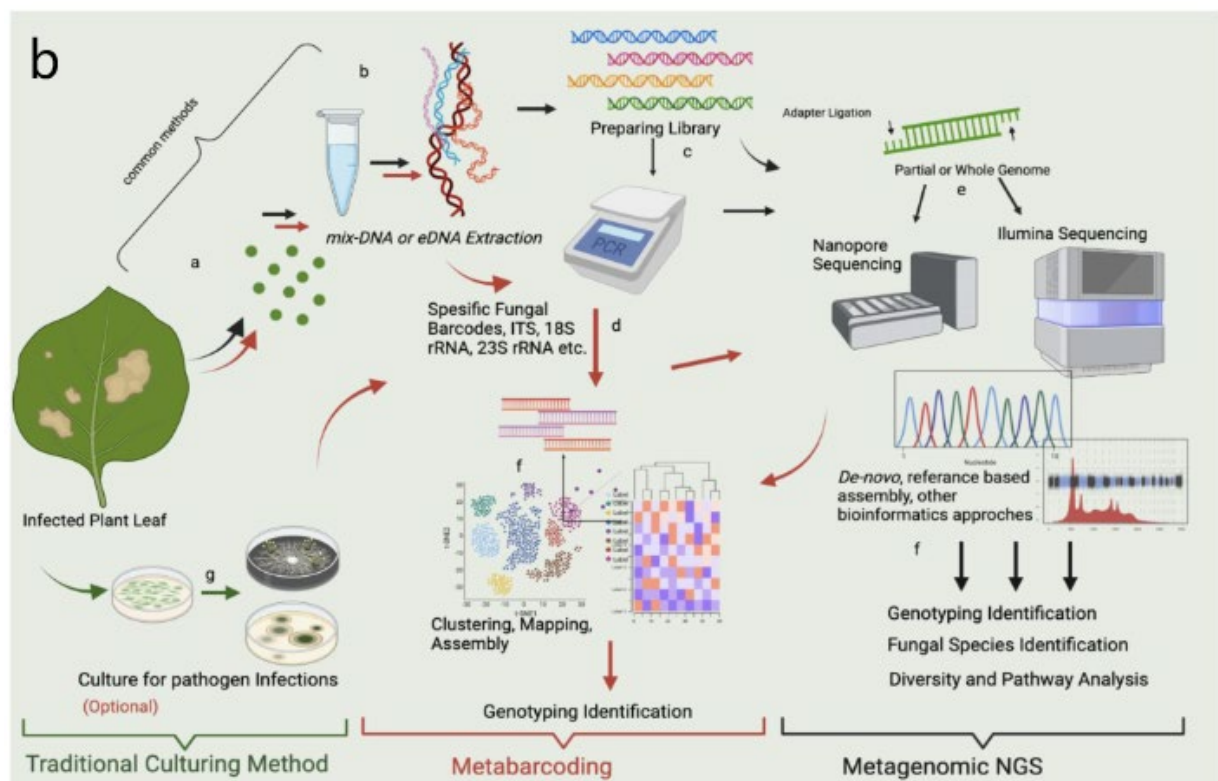
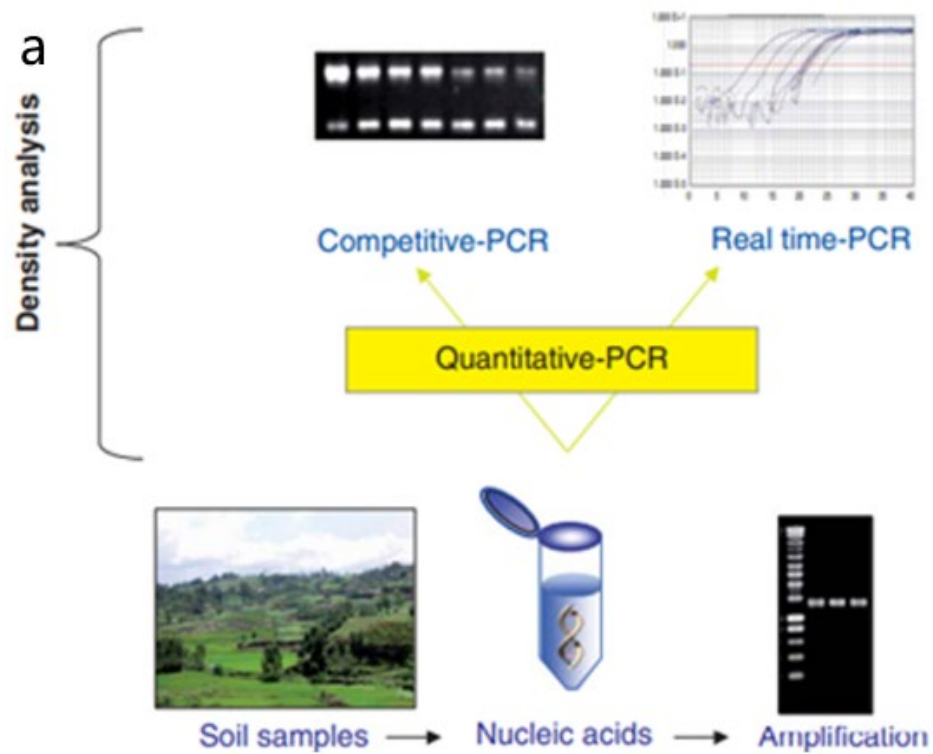


Figure 1-11 Methods used to assess diversity and density of denitrifiers with a PCR-based approach: a) qPCR (Philippot et al. 2007) and b) metabarcoding and metagenomic (Gökdemir et al. 2022).

Despite of PCR and qPCR, Fluorescence in situ hybridization (FISH) and 16S rRNA sequencing analysis are also used to study the genetic diversity of denitrifiers. FISH is a molecular cytogenetic technique that uses fluorescent probes that bind to only particular parts of a nucleic acid sequence with a high degree of sequence complementarity. It was developed to detect and localize the presence or absence of specific DNA sequences on chromosomes. Gomez-Villalba et al used PCR to amplify a partial sequence of the 16S rRNA gene, and then analyzed the microbial diversity on biological treatment membranes in sewage plants. Analysis of the *nosZ* and revealed the presence of denitrifying bacteria in both the aerobic and anaerobic regions of the water treatment system (Revsbech et al. 1988).

### **2.3. Sediment – a hotspot for denitrification**

Soils and sediments are a favorable habitat for microbes. Particularly riverine sediments provide an excellent anoxic environment for denitrifiers. Water saturation restricts O<sub>2</sub> penetration into sediments to less than several centimeters, below which organic carbon molecules must be broken down hydrolytically with nitrate as the primary electron acceptors.

The removal of nitrate in water is mainly based on the process of assimilation and denitrification. Denitrification, most of it occurring in sediments, accounts for a large fraction of organic carbon mineralization at the land-ocean transition, with contributions of up to 40% in some polluted estuaries (Joergensen & Soerensen 1985). Therefore, the denitrification process plays a significant role on the removal of nitrate. Denitrification contributes differently to the removal of nitrate in different environmental circumstances. Studies have shown that it can be as high as 63% in some high nitrogen content water bodies (Berelson et al. 1998, Lin et al. 2009). Exploring denitrification in sediments is helpful to understand the nitrogen removal process as part of ecological restoration. Denitrification rates are greatly affected by factors such as temperature, nitrate concentration, organic matter content, and redox conditions (Baeseman et al. 2006, García-Ruiz et al. 1998). In addition, the presence of submerged plants can change the physical and chemical conditions of the surrounding environment and indirectly affect denitrification as well.

#### **2.3.1. Denitrification conditions in the environments**

##### **Nitrate**

Nitrate concentration is the main factor affecting denitrification rates, especially in aquatic

ecosystems (Esteves et al. 2001, García-Ruiz et al. 1998, Magalhaes et al. 2005). Typically, under denitrifying conditions, denitrification rates increase with increasing nitrate concentrations. In experiments where variable nitrate concentrations were supplied, the nitrate reduction rate (NRR) and N<sub>2</sub>O production rate (also referred to as denitrification rate, DNR) were fitted to the Michaelis-Menten rate expression:

$$R = \frac{R_{\max} [\text{NO}_3^-]}{K_m + [\text{NO}_3^-]}$$

where R stands for either DNR or NRR,  $R_{\max}$  is the maximum DNR or NRR,  $K_m$  is the affinity constant for NO<sub>3</sub><sup>-</sup> and [NO<sub>3</sub><sup>-</sup>] is the average nitrate concentration in the reactor (Laverman et al. 2006).

### **Carbon source**

In the biochemical denitrification process, the carbon sources that can be used for denitrifying bacteria are mainly natural organic carbon (Bijay-singh et al. 1988, Foglar & Briški 2003, Stanford et al. 1975). Organic carbon is the electron donor in the denitrification process, its can affect the denitrification rate (Baeseman et al. 2006, Pfenning & McMahon 1997). The commonly used external carbon sources in bacterial culturing are glucose and acetate, as easily degradable carbon sources allowing a high denitrification rate. The low organic carbon content in the sediment can limit the denitrification rate (Bradley et al. 1995, Morris et al. 1988). However, the denitrification rate is not limited by organic carbon in the surface sediments, likely due to their relatively high organic carbon content (Pfenning & McMahon 1997).

### **pH**

The pH value is an important influencing factor of the denitrification process. The most suitable pH range for denitrifying bacteria is 6.5-7.5 when pure culturing (Nguyen et al. 2018, Oh et al. 2000). When the pH value is not in this range, the denitrification rate decreases significantly. In general, denitrifying bacteria will be inhibited when the pH value is over 9.5. If the pH value of the sediment is between 7 and 8, it will be suitable for denitrification. In other words, the weak alkaline sediments' environment will not cause significant restriction on denitrification.

### **Dissolved oxygen**

Most of the denitrifying bacteria are facultative anaerobic bacteria. Only in the absence of molecular oxygen, denitrifying bacteria will use nitrate or nitrite to breathe and reduce nitrogen atoms. If the dissolved oxygen concentration is too high, molecular oxygen becomes an oxygen-supplying substance, which will promote the aerobic metabolism of denitrifiers (Oh & Silverstein 1999) and inhibit the reduction of nitrogen nitrate (Holman & Wareham 2005, Robertson & Kuenen 1984). The ambient oxygen restricts the synthesis step of denitrification enzymes (Sirivedhin & Gray 2006). However, complete anaerobic conditions in the sediment will inhibit the denitrification intensity, by reducing the generation of substrates such as nitrate and nitrite required for denitrification, this is because if there is no nitrate from the overlying water, oxic conditions are required for nitrification and nitrate producing. Therefore, ensuring the right amount of oxygen can help improve the denitrification process (Knowles 1984). The oxygen in sediments mainly comes from overlying water. Therefore, strengthening the aeration of the bottom body of the pond is of great significance for increasing the intensity of denitrification in the sediment, reducing the concentration of toxic nitrogen, and promoting the nitrogen cycle.

### **Temperature**

The rate of denitrification increased rapidly with rise in temperature from 2° to 25 °C (Bremner & Shaw 1958). When the temperature is lower than 15 °C, the denitrification rate will be reduced significantly. Reports on denitrifying sediments show that the denitrification rate increases exponentially with increasing temperature, such as lake sediments in the range of 14-35.5 °C (Messer & Brezonik 1983), and marine sediments in the range of 5-18 °C (Sybil et al. 1984). The same phenomenon was also found in river sediments within the range of 5-25 °C (García-Ruiz et al. 1998). However, once the temperature exceeds this range, the denitrification rate will decrease. Therefore, denitrification rate tested in the low-temperature winter season could be much lower than that in summer.

### **Contaminants**

Some contaminants, such as heavy metals and organic pollutants, can inhibit the growth and activity of denitrifying microorganisms, leading to a decrease in denitrification rates. However, other contaminants may also stimulate denitrification under certain conditions. For example, organic carbon and electron acceptors such as sulfate and iron can stimulate denitrification under some circumstances.

Antibiotics are also environmental contaminants. Antibiotics can have various effects on denitrification in the environment, depending on the specific type of antibiotic, the concentration of the pollutant, and the characteristics of the environment in which denitrification is occurring. In general, antibiotics can inhibit the growth and activity of the microorganisms involved in denitrification, which can lead to a reduction in the rate of denitrification (Zhang et al. 2019). This is because denitrification is carried out by a complex community of microorganisms, including bacteria and fungi, and some of these may be sensitive to antibiotics. Moreover, antibiotic pollutants can also disrupt the microbial communities involved in denitrification by changing the balance of different species, which can lead to a decrease in denitrification efficiency (Fan et al. 2020). The effects of different antibiotics on denitrification have been summarized in Table 1-7 and discussed (section 1.2.3).

### **2.3.2. Denitrification measurements in sediments**

A wide range of experimental methodologies have been developed to estimate denitrification rates in sediments, including N-input and output mass balance methods (Lomstein et al. 1998), N<sub>2</sub> production measurements (Nowicki 1994), the acetylene inhibition technique (Yoshinari & Knowles 1976), nitrate consumption measurements (Andersen 1977), diagenetic models of pore water profiles (Seitzinger et al. 1984) and <sup>15</sup>N-tracer techniques (Nishio et al. 1983). The acetylene inhibition technique (AIT) is based on the inhibition of nitrous oxide reductase by acetylene and is a simple, sensitive and inexpensive method. However, it suffers from several disadvantages, notably that at the low nitrate concentrations often found in marine sediments (< 10 µM) inhibition is incomplete, and in marine sediments sulfide reverses the acetylene block of nitrous oxide reductase activity (Knowles 1990). As a consequence of these experimental limitations, direct measurements using <sup>15</sup>N-tracer techniques are now the preferred method for measuring denitrification rates. The recently developed <sup>15</sup>N isotope pairing method is a powerful analytical technique which not only enables the rate of denitrification to be accurately determined but also the proportion arising from nitrate in the water column and that produced by nitrification in the sediment. This experimental approach has the major advantage that no inhibitor is required and that nitrification, denitrification, nitrate ammonification and N-mineralisation rates can all be determined in a single experiment if the nitrate and ammonium fluxes have been measured (Rysgaard et al. 1996).

Since nitrate reductase activity (Abdelmagid & Tabatabai 1987) and denitrification intensity (Yu

et al. 2008) measures the reduction of nitrate and total nitrogen, respectively, these measurements in the sediment can also be used to determine the denitrification rate. Denitrification potential (Yeomans et al. 1992) and denitrification enzyme activity (Smith & Tiedje 1979) are two concepts often used in the determination of denitrification. Denitrification potential refers to the amount of denitrification products ( $\text{N}_2\text{O}$  and  $\text{N}_2$ ) produced by denitrifiers (or samples containing denitrifiers) in a certain period of time when nitrate is added. It reflects the ability of living microorganisms in the sample which are capable of denitrification. It can be used to measure the potential for denitrification or the maximum rate of denitrification that a sample may have. No substances that inhibit the normal growth of microorganisms are added during the cultivation process, which is different from the determination of denitrification enzyme activity. Denitrification enzyme activity is an indicator for the enzyme activity in a sample. In order to prevent the growth of microorganisms, the method of adding low concentration of chloramphenicol is often used to inhibit the growth of microorganisms, and the denitrification enzyme activity is determined by measuring the generation rate of denitrification products. A concentration lower than  $0.125 \text{ mg g}^{-1}$  soil of chloramphenicol does not inhibit the denitrifying enzyme activity of microorganisms (Murray & Knowles 1999).

It is very difficult to study the ecology of microorganisms in the sediments of lakes and other water systems. The first difficulty is the sampling error. Grab sampler or core sampler can be used for surface sediment sampling. The sampling of the sediment must be based on the purpose of sampling and taking the characteristics of the sediment, pollutants and the surrounding environment of the site into account. The sediment is a very heterogeneous ecosystem, and the sediments on the banks, open water areas, lake centers and other places are different. Studies on denitrification in terrestrial soils at same altitude have shown that denitrification could have up to 10% differences (Parkin 1990). Even in the sediment of the same water area, the denitrification and the number of microorganisms from different sampling points may be different. Therefore, increasing amount of researches has been done on the ecology of denitrifiers in the sediment under water (Tiedje 1988). However, in general, for the sediments in the same water area, there are still some common features that are used as comparison targets. For example, the seasonal systemic changes in temperature caused by the season have a wide-ranging impact on denitrifiers at all locations. Avoiding the differences between the samples and taking their commonality should be a research method for samples

with large differences. Another big problem that plagues ecologists is how to obtain those microorganisms that actually complete a certain transformation in nature, rather than other microorganisms that grow faster under artificially set culture pressure (Herbert & Nedwell 1990a).

For the first question, Parkin et al 1987 suggested that the patchy distribution of particulate organic material is a significant factor influencing the magnitude and variability of natural denitrification rates in soil (Parkin 1987). The high variability associated with estimates of natural soil denitrification rates may be a direct result of patchy distributions of denitrification activity in soil. Another factor that can influence the denitrification rate distribution was soil O<sub>2</sub> concentration, caused by the distribution of anaerobic zones in soil aggregates (Leffelaar 1979, Smith 1980). The second issue is the study of microbial separation methods. Herbert and Nedwell found that several environmental factors (Herbert & Nedwell 1990b), like temperature, nutrient, oxygen and substrate concentration, could regulate nitrate respiration in intertidal sediments, by changing the substrates affinity of microbial (Nedwell 1999). Therefore, they suggested to separate target microbial by adjusting the cultivation factors.

Rates of microbial processes in sediments are traditionally measured using slurry incubations (Murray et al. 1989, Oremland et al. 1984, Oren & Blackburn 1979). Reaction kinetics in slurries may deviate significantly from those under in situ conditions, however, because homogenization and dilution of the sediments disrupts their physical, chemical and microbiological structure. In particular, the use of slurries may enhance microbial access to organic matter and electron acceptors, by removing substrate supply limitations due to diffusion (Marxsen & Fiebig 1993, Pallud & Van Cappellen 2006) and increasing the sediment surface area to solution volume ratio. As a result, sediment slurries generally overestimate microbial reaction rates (Kaspar 1982, Pallud & Van Cappellen 2006).

The appearance of a new type of N<sub>2</sub>O induction electrode has greatly promoted the study of denitrification in river bottom biofilms and sediments. This electrode can determine the concentration of N<sub>2</sub>O and O<sub>2</sub> in the millimeter range, and can be used to investigate the effects of nitrate concentration, organic matter, season, and light on denitrification in biofilms at the bottom of rivers (Revsbech et al. 1988).

An approach called flow-through reactors (FTR, see Figure 1-12) was designed to preserve the structural, geochemical and microbiological integrity of the sediment sections and, hence,

retrieve reaction parameters that apply to *in situ* conditions (Pallud & Van Cappellen 2006, Roychoudhury et al. 1998). Because soluble reactants are continuously added with the inflow solution and soluble products are continuously removed with the outflow solution, (near-) steady-state conditions can be reached in FTR experiments. In contrast, initial rates, rather than steady-state rates, are measured in slurry incubations. FTRs are therefore well-suited to measure kinetic parameters of microbially mediated processes, and to study the response kinetics of these microbial processes to environmental perturbations, in particular, compositional changes of the aqueous medium.

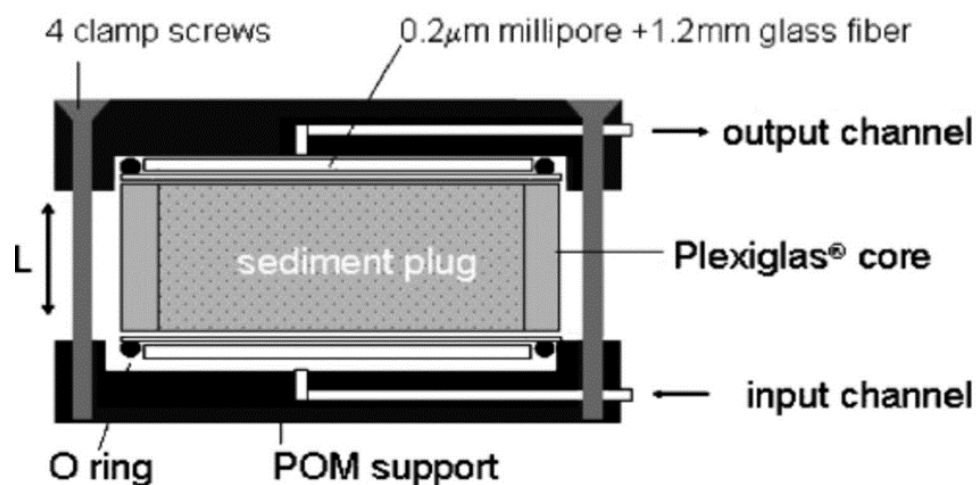


Figure 1-12 Side view of a flow-through reactor containing a sediment slice (Laverman et al. 2006).

### 2.3.3. The relevance of denitrification in the environment

As already discussed earlier, denitrification is of is a key process in the nitrogen cycle. It allows to reduce the amount of  $\text{NO}_3^-$  flowing into the river and the ocean due to leaching from the soil. Together with anammox, it forms a way for nitrogen in nature to return to the atmosphere (Barton et al. 1999, Hamilton 2007).

In agricultural production (Butterbach-Bahl & Dannenmann 2011), denitrification reduces nitrate to nitrogen, which reduces the nitrogen nutrient content in the soil and is not good for agricultural production. In agriculture, cultivating loose soil is often performed to prevent denitrification, because loose soil can provide oxic soil circumstance which inhibits anoxic denitrification process. Denitrification can cause huge losses of nitrogen fertilizers. From a global estimate, the nitrogen lost by denitrification is approximately equivalent to the amount of nitrogen fixed by biology and industry. On the other hand, denitrification is playing an important role in the world nitrogen balance.

In terms of environmental protection, denitrification and nitrification reactions together can constitute different technological processes, which are the main methods of biological nitrogen removal and are widely used in wastewater treatment plants worldwide (Keeney et al. 1971, Pinay et al. 2010, Xu et al. 2013). The use of nitrification and denitrification to reduce nitrogen pollution and eutrophication into rivers has become the consensus of environmental scientists.

### 3. Aquatic sediments

#### 3.1. Sediment: what and where?

Sediments are the result of weathering of rock, erosion and transportation remove the rock particles and transport them. Once these rock particles are deposited, it is called "sediment". If sediments get buried deep enough and undergo compaction and cementation, they turn into "sedimentary rocks".

Sediments are considered as one of the most active surfaces and interfaces, both physical and chemical, on the earth's surface area. Sediments are found in lakes, rivers, sand dunes, beaches, and the ocean. Most sediments are transported by water (fluvial processes), but also wind (aeolian processes) and glaciers. Beach sands and river channel deposits are examples of fluvial transport and deposition, though sediment also often settles out of slow-moving or standing water in lakes and oceans. Desert sand dunes and loess are examples of aeolian transport and deposition. Glacial moraine deposits and till are ice-transported sediments.

Sediments can be classified based on their grain size or composition. Grain size (or particle size) is the diameter of individual grains of sediment, or the lithified particles in clastic rocks. This is different from the crystallite size, which refers to the size of a single crystal inside a particle or grain. A single grain can be composed of several crystals. Granular material can range from very small colloidal particles, through clay, silt, sand, gravel, and cobbles, to boulders. Sediment grain size is measured on a log base 2 scale, called the  $\phi$  scale, which classifies particles by size from "colloid" to "boulder" (Krumbein 1934).

The organo-mineral composition in sediments could be very different from various conditions (Bock & Mayer 2000, Kögel - Knabner et al. 2008, Patience et al. 1996). In nitrogen-rich environments, microorganisms produce and recycle most organic matter, from which degraded particles are deposited onto the underlying water floor. In sediments, plant products are degraded extensively by microorganisms, leaving small organic remnants. Much of the surviving organic matter is intimately associated with mineral surfaces and enclosed within particle aggregates, and thus may be physically protected from microbial attack. As a result of oxidation and degradation under oxic conditions, even physically protected organic matter can slowly be mineralized, along with intrinsically resistant substrates such as lignin, pollen, kerogen and coal. Thus the only long-term shelter from mineralization is within anoxic

sediments. Although typically present at concentrations lower than 10% (in weight), nonliving organic materials strongly affect the physical and chemical properties of the sediments in which they occur. Not surprisingly, sedimentary organic substances have been the subject of active scientific investigation for over 100 years (Hedges & Oades 1997, Henrichs 1995). Sediment composition can be determined in terms of parent rock lithology, mineral composition or chemical make-up.

### **3.2. Minerals in sediments**

Sediments consist of different types of minerals. Common sedimentary minerals include quartz, feldspar, mica, clay minerals, and carbonates such as calcite and dolomite (Bayliss 1986, Curtis & Spears 1968, Daneshvar & Worden 2018, Herron & Matteson 1993). These minerals provide valuable information about the depositional environment of the sediment, including the source of the sediment, the type of sedimentary basin, and the conditions under which the sediment was deposited.

Iron oxides and manganese oxides are important minerals that are commonly found in sediments. These minerals play a significant role in the formation and preservation of sediments, as well as in environmental processes. Iron oxides, such as hematite ( $\text{Fe}_2\text{O}_3$ ) and goethite ( $\text{FeO}(\text{OH})$ ), are formed through the oxidation of iron-bearing minerals like pyrite ( $\text{FeS}_2$ ), in the presence of oxygen and water (Hasselov & von der Kammer 2008). Iron oxides can impart a distinctive red or yellow color to sediments, and they are also important for their ability to adsorb and trap trace elements and nutrients. Manganese oxides, such as birnessite ( $\text{Na}_{0.7}\text{Ca}_{0.3}\text{Mn}_3\text{O} \cdot 2.8\text{H}_2\text{O}$ ) and vernadite ( $\text{MnO}_2 \cdot n\text{H}_2\text{O}$ ), are also commonly found in sediments, particularly in marine environments (Anschutz et al. 2005, Turner 2000). Manganese oxides are often formed through the oxidation of dissolved manganese in seawater, and they can form in a variety of morphologies, including as coatings on sediment grains, as nodules, or as dendritic aggregates. Manganese oxides play an important role in regulating the cycling of metals and nutrients, due to their high chemical reactivity.

#### **3.2.1. Iron oxides**

Iron (Fe) is one of the most abundant elements (6%) of the earth's crust. On our planet, iron is ubiquitous in the hydrosphere, lithosphere, biosphere and atmosphere, either as particulate ferric [ $\text{Fe}(\text{III})$ ] or ferrous [ $\text{Fe}(\text{II})$ ] iron-bearing minerals or as dissolved ions (Kappler & Straub

2005). Redox transformations of iron, as well as dissolution and precipitation and thus mobilization and redistribution, are caused by chemical and to a significant extent by microbial processes.

Names and formulas of some important iron minerals are listed below as Table 1-13. Fe oxides (hematite, magnetite), oxyhydroxides (goethite, akaganeite, lepidocrocite, feroxyhyte) and hydrous oxides (ferrihydrite, hydrohematite, maghemite) [together abbreviated 'FeO<sub>x</sub>'] have important roles in biogeochemical processes at the Earth's surface (Waychunas et al. 2005). FeO<sub>x</sub> occur in the atmosphere, pedosphere, biosphere, hydrosphere, and lithosphere in a great diversity of morphologies, minerals, and associations (Braunschweig et al. 2013). They are characterized to have wide occurrence, tendency to nucleate and grow on the surfaces of other phases, important redox capabilities, and relatively high reactivity. Fe is commonly leached from minerals by both inorganic weathering processes and biological activity (Barker et al. 1998), resulting in the concentrated FeO<sub>x</sub> phases found in nearly all surficial soils and sediments (White & Brantley 2018).

Table 1-13 Names and formulas of some important iron minerals (Kappler & Straub 2005)

Fe(III) oxides	Fe(III) oxyhydroxides and hydroxides
Hematite $\alpha$ -Fe <sub>2</sub> O <sub>3</sub> Maghemite $\gamma$ -Fe <sub>2</sub> O <sub>3</sub>	Goethite $\alpha$ -FeOOH Lepidocrocite $\gamma$ -FeOOH Ferrihydrite Fe <sub>5</sub> HO <sub>8</sub> ·4H <sub>2</sub> O
Fe(II) minerals	Mixed Fe(II)-Fe(III) minerals
Ferrous monosulfides FeS Pyrite FeS <sub>2</sub> Siderite FeCO <sub>3</sub> Vivianite Fe <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>	Magnetite Fe <sub>3</sub> O <sub>4</sub> Green rusts Fe <sub>x</sub> <sup>III</sup> Fe <sub>y</sub> <sup>II</sup> (OH) <sub>3x+2y-z</sub> (A <sup>-</sup> ) <sub>z</sub> ; A = Cl; SO <sub>4</sub> Greigite Fe <sub>3</sub> S <sub>4</sub>

One of the main processes for FeO<sub>x</sub> phases generation in the environment is the oxidation of dissolved iron. Leached Fe(II) in reducing, anaerobic conditions has a relatively high solubility in pH 7 waters, with the saturation concentration of the Fe(II) hexaaquo species approaching 0.5 M at room temperature. However, dissolved Fe concentrations drop below 10<sup>-12</sup> M if the Fe(II) is oxidized to the hexaaquo Fe(III) or polymerized species (Mesmer & Baes 1976). This reaction, which occurs when ground water reaches the surface (or due to mixing of subsurface fluids) and abundant dissolved O<sub>2</sub>, results in the precipitation of FeO<sub>x</sub> and is commonly observed at natural springs. Iron oxyhydroxides can also form under anaerobic conditions, for example, when iron oxidation is coupled to nitrate reduction (Straub et al. 1996). This reaction, and iron oxidation in microaerophilic environments, is often catalyzed by microorganisms.

Consequently, the mineral products are frequently associated in the environment with cell surfaces and extracellular polymers (Chan et al. 2004).

Another process generating  $\text{FeO}_x$  is the oxidation of pyrite ( $\text{FeS}_2$ ). In pyrite-rich rocks such as those associated with ore deposits, this can lead to environmental contamination known as acid mine drainage. Oxidation of sulfide generates solutions that are quite acidic (pH 2 or less) and that carry high concentrations of both dissolved  $\text{Fe(II)}$  and  $\text{Fe(III)}$ . The reaction generates aqueous  $\text{Fe(II)}$ , and  $\text{Fe(III)}$  is formed by reaction with dissolved oxygen. However, low solubility of  $\text{O}_2$  in these liquids and the slow rate of inorganic oxidation from ferrous ion to ferric ion by oxygen allow  $\text{Fe(II)}$  concentrations on the order of 0.5 M. The  $\text{Fe(II)}$  can be oxidized by mixing of mine waters with surface oxygenated water, or catalyzed by microbial activity (Edwards et al. 2000) to produce large quantities of nanoparticulate  $\text{FeO}_x$ .

### **3.2.2. Adsorption ability of iron oxides**

Sorption processes on  $\text{FeO}_x$  phases are due to the formation of chemical bonds with surface atoms, a process known as chemisorption. The bonds can be covalent, ionic, or mainly involve hydrogen bonds. Though there is variation in the bonding nomenclature used in the literature, we can speak generally of inner-sphere and outer-sphere types of surface complexes that are chemisorbed. Inner-sphere complexes share some of their first coordination shells with surface oxygen or anionic species. For cations the shared atom is in the ligation sphere of the cation, while for anions it would involve the oxygens of the anion itself. Outer-sphere complexes are bound to a surface by weaker electrostatic attraction and have intervening water molecules between themselves and the surface. They are thus farther from the sorbing surface. One can readily imagine intermediate cases of binding by hydrogen bonds that are weaker than inner-sphere binding, but stronger than outer-sphere binding.

Due to its strong binding ability to organic compounds, sedimental iron oxides are widely studied in riverine pollution cases, such as antibiotic pollution. For example, Gu and Karthikeyan (Gu & Karthikeyan 2005) found that adsorption of ciprofloxacin (CIP) onto iron hydrous oxides was strongly pH-dependent, and CIP could form a six-membered ring with Fe on the iron hydrous oxide surface. Zhao et al (Zhao et al. 2014) also claimed that pH greatly affected the tetracycline (TC) adsorption on goethite. Tan et al (Tan et al. 2015) demonstrated that metal cations and fulvic acid could enhance the CIP adsorption onto goethite through cation bridging and conversion of the goethite surface to a lipophilic surface, respectively. A

study on the interaction of oxolinic acid (OA) with synthetic goethite ( $\alpha$ -FeOOH) and akaganeite ( $\beta$ -FeOOH) proved the importance of the iron triggered surface complexation mechanism in facilitating a mononuclear six-membered chelate complex (Marsac et al. 2016). In the case of sulfadiazine, iron coating inhibited its transport in quartz sand through hydrophobic effect, complexation, and electrostatic attraction. An increase in surface roughness and specific surface area of quartz sand, after iron oxides load, also increased retention of SDZ in column (Zhang et al. 2021).

### **3.2.3. manganese oxides**

In the Earth's crust, manganese (Mn) is the 10<sup>th</sup> abundant element and the 2<sup>nd</sup> common heavy metal following iron, which is an essential element for the nutrition of plants and animals (Post 1999, Tebo et al. 2004). Near the Earth's surface, Mn is easily oxidized, giving rise to more than 30 known manganese oxides (referred to generally as Mn-oxides, including oxides, hydroxides and oxyhydroxides) (Burns & Burns 1977, Post 1999), which are ubiquitous in a wide range of natural environmental settings, such as ocean floors, soils and sediments, desert varnishes, and fresh-water bodies (Post 1999). These Mn-oxides possess a number of superior physical and chemical properties, such as high structural flexibility and stability, fast cation diffusion under high charge–discharge rates and environmental friendliness (Thackeray et al. 2005, Wei et al. 2011).

In natural systems, Mn-oxides are not only vital components, but also exhibit versatile functions in natural redox reactions, microorganism distribution and the biogeochemical cycle (Shindo & Huang 1982). Actually, as very strong oxidizing agents and versatile minerals, Mn-oxides have been received much attention because it can participate in a variety of chemical and biological reactions, thereby mediating the fate and transport of numerous contaminants and nutrients, further affecting the water quality of marine and soil systems. Therefore, Mn-oxides have been used in broad applications such as catalysts, sorbents, sensors, magnetic materials with a perovskite structure, energy storage (i.e., supercapacitors), chemical conversions (i.e., water splitting and oxygen evolution reaction) and electrode materials, mainly due to their high redox potential, environmental friendliness and low cost (Liu et al. 2013, Xu et al. 2017, Zhu et al. 2020).

Layer structured Mn-oxides are believed to be the major existing form of manganese minerals in nature, and it tends to be more reactive than those with tunnel structure due to the impact

of the mineral configuration on surface area, vacancy and edge sites (Post 1999). Birnessite (structure see Figure 1-14), as one of the most common Mn-oxides with a layered structure, is a major phase in several soils and nodules, as well as an alternative product in manganese rich ores. It also has various industrial and commercial applications, including as a good ion exchanger, controlling heavy metals and other trace metals in soil and groundwater.

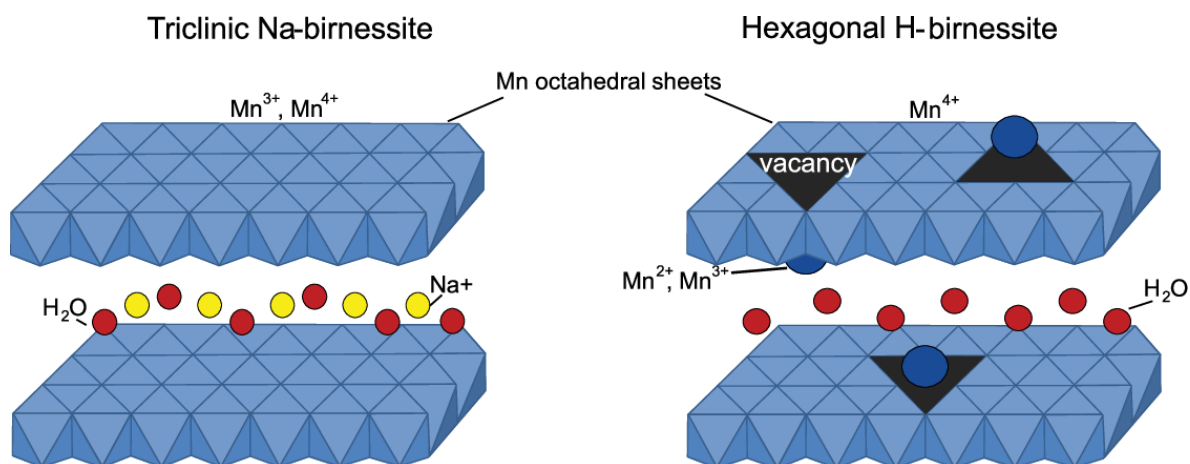


Figure 1-14 Schematic diagram of triclinic Na-birnessite and hexagonal H-birnessite (Lanson et al. 2000).

### 3.2.4. Role of birnessite in the degradation of antibiotics

Birnessite occurs extensively as fine-grained and relatively poorly crystalline phases in soils and sediments and/or as mineral coatings (Cygan et al. 2012, Post 1999, Zhao et al. 2016). Due to its extraordinary adsorption and oxidation capacities, birnessite is known to play an important role in metal ion dynamics, nutrients and carbon cycling, elements and the fate of a wide variety of contaminants from the aqueous environment (Islam et al. 2018, Yang et al. 2021).

Birnessite in sediments is reactive surface that plays an important role in affecting the fate and transport of organic contaminants, for example fluoroquinolones (FQs). The redox reaction between FQs and birnessite is initiated by generating a surface complex, then one electron is transferred from FQ to the surface-bound Mn(IV), followed by oxidation of FQ's piperazine moiety to generate a radical intermediate, and to yield Mn(III) that can be further reduced to Mn(II) (shown in Figure 1-15). The formed radical intermediates subsequently undergo N-dealkylation, C-hydroxylation, and possibly coupling to yield a range of products (Zhang & Huang 2007). The final organic products and Mn(II) may be released from the oxide surfaces. The surface complex formation and electron transfer step may be rate-limiting. In addition,

the concentration of dissolved Mn(II) was considerably lower than the loss of organic compound at any given time, most likely due to strong adsorption of Mn(II) ions to MnO<sub>2</sub> surfaces (Zhang &Huang 2005).

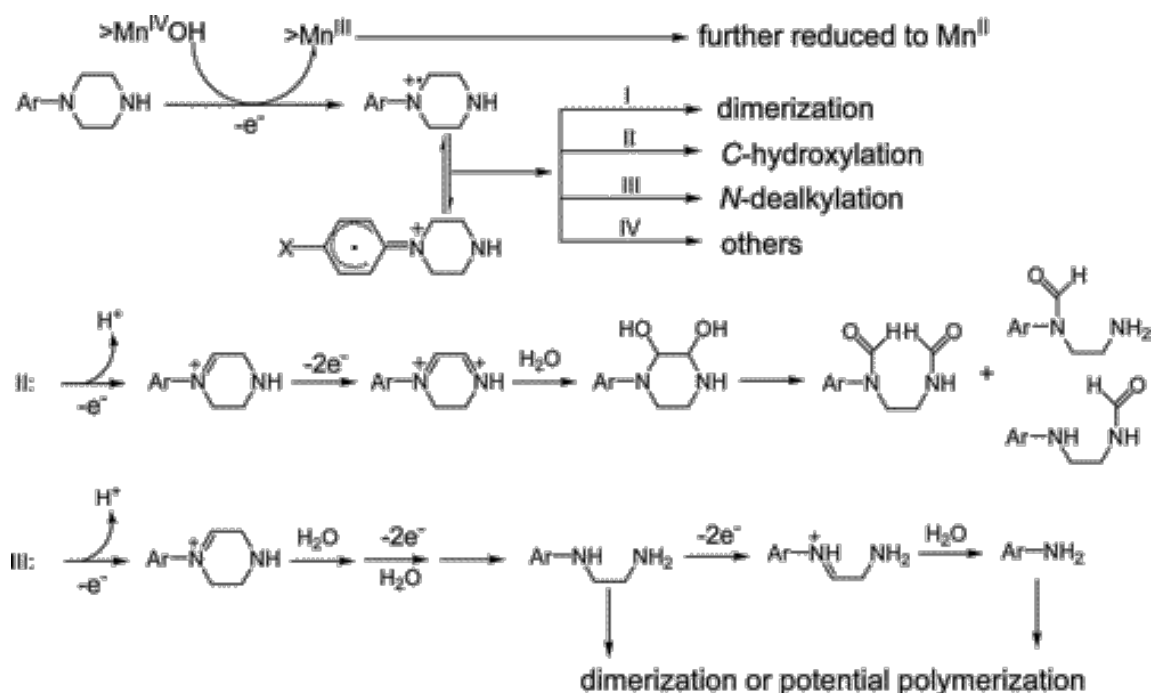


Figure 1-15 Proposed reaction scheme for oxidation of FQ's piperazine moiety by MnO<sub>2</sub> (Zhang &Huang 2005)

A series of FQs are highly susceptible to oxidation by birnessite in the following order, from fastest to slowest: CIP ~ enrofloxacin (ENR) ~ norfloxacin (NOR) ~ ofloxacin (OFL) > lomefloxacin (LOM) > piperacillin (PIP) (Zhang &Huang 2005). The relative reaction order corresponds to the ability of the compounds to adsorb on the oxide surface. These zwitterionic (a molecule having a net formal charge of zero, but negative and positive formal charges on individual atoms within its structure) compounds adsorb more strongly and are oxidized faster under acidic conditions. The piperazine moiety of the FQs is involved in adsorption and oxidation. The results of chemical reactivity and product identification among a wide range of FQs show that the FQ's piperazinyl N atoms are the primary adsorptive and oxidative sites toward MnO<sub>2</sub> (Zhang &Huang 2007). Flumequine, a structurally similar compound that lacks a piperazine ring, is unreactive with MnO<sub>2</sub> (Zhang &Huang 2005).

Denitrifying bacteria are important in sediment as they play a key role in the nitrogen cycle. However, the presence of antibiotic pollutants can have a negative impact on these bacteria, leading to a disruption in the natural balance of the ecosystem. This disruption can be further compounded by the presence of minerals like iron oxides and manganese oxides, which can

adsorb these pollutants and impact their availability to denitrifying bacteria. Understanding these interactions is important for managing sediment ecosystems and preventing negative impacts on the natural balance of the system. One key aspect that is explored in depth is the interaction between denitrifying bacteria, antibiotic pollutants, and minerals like iron oxides and manganese oxides that are found in sediments.

#### **4. Antibiotics in the environment: impact of metal oxides and effect on denitrification**

Environmental contamination by antibiotics is of increasing interest because of their ability to select antibiotic resistance. Because of their specific action on bacteria, a risk of disturbance of biogeochemical cycles dominated by bacterial activity may exist. The presence of antibiotics in sediments, the important compartment for denitrification and the impact on this process can thus be questioned. Some recent studies have investigated the effects of low continuous concentrations of antibiotics on the process of denitrification (DeVries et al. 2020, DeVries et al. 2015). The impact of antibiotics on denitrifiers depends on the availability, form and speciation of the antibiotics in the sediments, which depends on the composition of the sediment notably its minerals. Metal oxides present in sediment could interfere with antibiotics by sorption and/or degradation. Meanwhile, the presence of minerals in sediments may also have important implications for microbial processes, including denitrification. Iron and manganese oxides are known to be potential electron acceptors in denitrification, and their presence in sediments might influence the rate and extent of denitrification. Recent studies have shown that metal oxides can adsorb antibiotics and promote their degradation processes (Li et al. 2022, Martin et al. 2015, Zhou et al. 2019). Especially in sediments, where antibiotics can accumulate, and where the contact time between bacteria and antibiotics are high, the effect of antibiotics on environmental beneficial bacteria like denitrifiers needs to be studied as well as the interactions with the mineral and organic phases of the sediment. This highlights the complex interactions between minerals, microorganisms, and anthropogenic contaminants in natural sediment systems (Figure 1-16). Understanding these interactions is essential for developing effective strategies to manage and mitigate the impact of pollution on sedimentary environments.

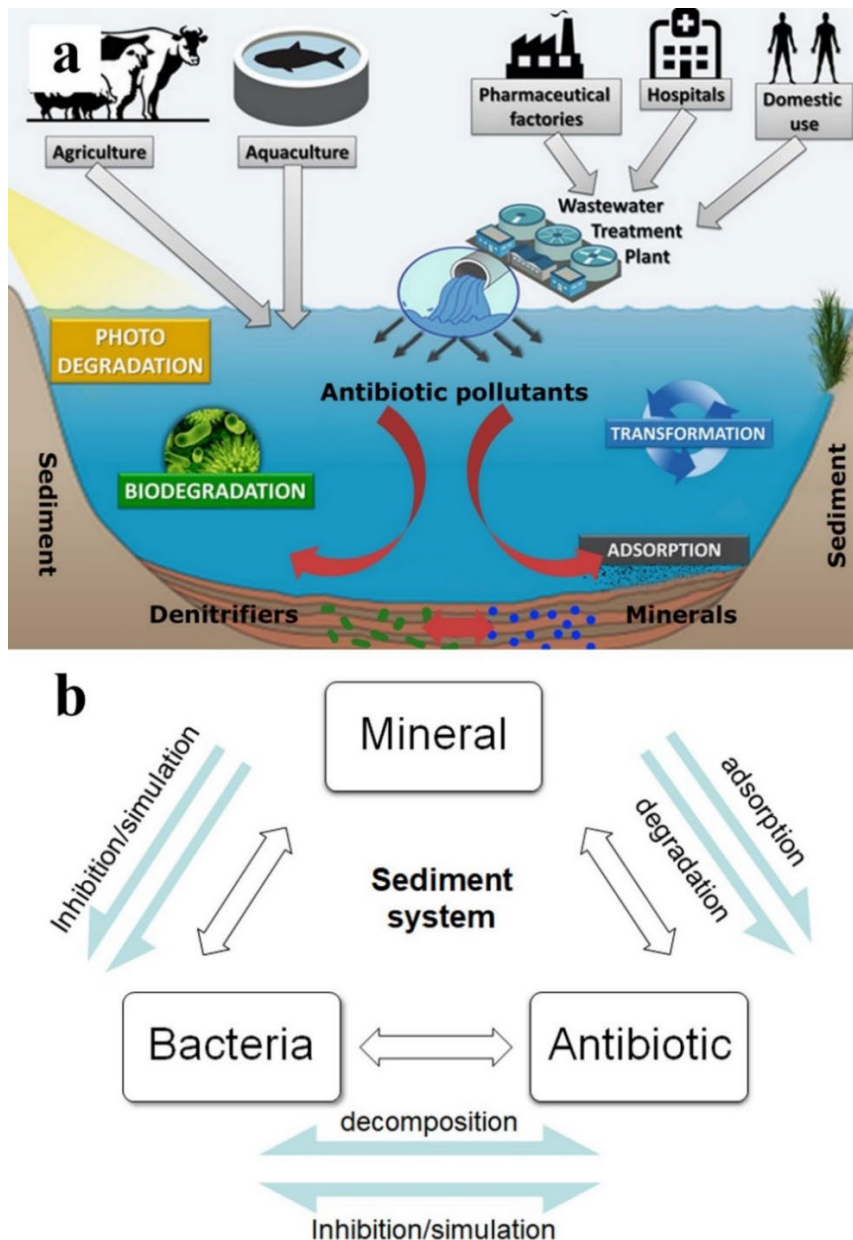


Figure 1-16 a) The relationships of antibiotics, minerals and denitrifiers; b) Possible interactions between antibiotics, minerals and bacteria in the sediment system.

This interactions between antibiotics, minerals and bacterial activity are the basis of my PhD topic 'transfer of antibiotics at mineral interfaces: impacts on denitrification in aquatic sediments systems'. My thesis is composed of the following chapters related to this topic:

In **chapter 2** the effects of different antibiotics on the growth and denitrifying activity of an environmental bacterial are studied. The goal of this chapter was to answer the following questions:

- Are antibiotic resistance genes present in a non-pathogenic environmental denitrifying species?
- Is this species' performance consistent with the prediction by the antibiotic resistant genes found in the genome?
- Do antibiotics affect the growth and denitrifying activity of this species? How and why?

To this end, the antibiotic resistance of a denitrifying species, named *Pseudomonas veronii*, isolated from a river sediment was assessed. The denitrifier was tested towards 6 different antibiotics, covering different families and modes of action. As a gram-negative species, and due to its outer membrane characteristics, it was expected that this species would be resistant towards macrolide antibiotic erythromycin (ERY) and tylosin (TYL). In addition to macrolides, tetracycline (TC) and three quinolones (flumequine (FLU), ciprofloxacin (CIP) and ofloxacin (OFL)) were tested. In addition to cell wall properties, efflux pumps may allow intrinsic resistance, therefore their role was assessed by using an efflux pump inhibitor while *P. veronii* was exposed to the antibiotics. The antibiotic resistance was established by determining the minimal inhibiting concentrations, by a standard broth microdilution susceptibility method. In addition to this, the effect of these antibiotics on denitrifying growth and activity were investigated. Furthermore, the genome of the denitrifier was sequenced and examined regarding antibiotic resistance genes (ARGs).

In **Chapter 3** the effects of antibiotics and their degraded byproducts on denitrification activity on a denitrifying isolate *P. veronii* were investigated. The aim of this chapter was to answer whether

- What is the reaction pathway of antibiotic TC and CIP with birnessite? What byproducts can be found after react with birnessite?
- Do TC/CIP byproducts affect growth of *P. veronii*?
- Do TC/CIP by-products affect activity of *P. veronii*?

Representative antibiotics, ciprofloxacin (CIP) from fluoroquinolone family and tetracycline (TC) from tetracycline family, were used in this work. By controlling the exposure time of CIP/TC mother compound in birnessite contained solutions, we were able to obtain solutions with different proportions of byproducts. These mixtures with different compound proportions were then applied to the same denitrifier. The toxicity of CIP/TC and their birnessite-induced

byproducts was investigated under pure cultured conditions by a broth micro-dilution susceptibility method, and the effects of these compounds on denitrification process was assessed via closed bottle test. Different byproducts were hypothesized to have different effects on both growth and nitrite reduction activity of *P. veronii*. Consequently, we could give reasonable prediction on ecotoxicity of CIP/TC in mineral-rich water environment.

**Chapter 4** describes the transport and fate of tetracycline (TC) and ciprofloxacin (CIP) in complex coastal sediments in order to answer the questions:

- Can coastal sediment act as sink for TC and CIP?
- Can transformation or degradation of TC and CIP occur in coastal sediment?
- Do bacteria contribute to the removal of TC and CIP in sediments?

The sediments were characterized by XRD and chemical methods. Two types of experiments were carried out: 1) flow through reactors (FTR) to simulate the continuous flow of TC and CIP in an intact coastal sediment environment and 2) batch experiments with the sediments in order to check if biodegradation contributes to the total degradation of TC and CIP in sediment environment. The FTR were divided into 3 groups by different input solutions: TC only, CIP only and a mixture of TC and CIP. The outflow solution of the FTR was collected every day and was analyzed by HPLC to determine the antibiotic concentrations after going through the sediment. After 3-weeks, the sediment supplied with the different antibiotic was freeze-dried, extracted and then analyzed to determine the transformation of TC and CIP. The aim of this part is to determine whether coastal sediment can act as sink for antibiotics and if transformation of antibiotics can occur in these coastal sediments.

The PhD topic, "Antibiotics in the environment: impact of metal oxides and effect on denitrification", is expected to provide insights and contribute to addressing the urgent issue of presence of antibiotics and the antibiotic resistance in the environment. By investigating the interactions between antibiotics, minerals, and denitrifying bacteria, we can better understand the fate and transport of antibiotics in the environment, and develop strategies to mitigate their impact. In particular, our findings may inform the development of novel approaches for remediation of antibiotic-contaminated sediments and other environmental matrices. Ultimately, our research has the potential to inform policy decisions and promote sustainable management practices to preserve the health and integrity of our environment.

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## **Chapter 2**

### **Antimicrobial resistance in the riverine denitrifying *Pseudomonas veronii***

**In preparation**



## Abstract

We investigated the antibiotic resistance in a non-pathogenic, denitrifying bacteria *Pseudomonas veronii*. This isolate, from a river sediment, is capable of complete denitrification from  $\text{NO}_3^-$  to  $\text{N}_2$ . *P. veronii* was tested towards a range of antibiotics via the classical disc method as well as a cultivation method allowing to determine minimal inhibiting concentrations as well as dose-effect curves against 6 chosen antibiotics. In parallel antibiotic resistance genes in the genome of the denitrifier were identified via annotation.

*P. veronii* was resistant towards macrolide erythromycin (ERY) and tylosin (TYL), tetracycline (TC) and quinolone flumequine (FLU). On the other hand, *P. veronii* was susceptible towards quinolone ciprofloxacin (CIP) and ofloxacin (OFL). Resistance towards the large lipophilic antibiotics (ERY and TYL) is most likely related to the exclusionary properties of the cell envelope. The presence of efflux pump genes most likely explains resistance towards the other antibiotics tested. Under denitrifying conditions, exposure to TYL and TC slightly inhibited growth (less than 10%). ERY, FLU and OFL inhibited growth with respectively 17, 21 and 37% with the highest impact of CIP (55% inhibition). In the presence of TYL, with little impact on the growth of *P. veronii*, nitrate is reduced at a similar rate as the control, however nitrite reduction is stalled. The presence of the ERY, FLU, OFL, TC and CIP slow down nitrate reduction and all result in the accumulation of nitrite.

Our results show that the non-pathogenic denitrifier *P. veronii* exhibits resistance towards several antibiotics, however susceptibility towards others. The latter most likely affects the functioning of this species in the environment and its denitrifying activity which will be investigated in the future.

**Keywords:** antibiotic; denitrifier; genomic information; minimal inhibiting concentrations; denitrification activity

## 1. Introduction

The nitrogen cycle is one of the major biogeochemical processes on earth. Among nitrogen cycling processes, denitrification plays a key role in reducing inorganic nitrogen, as nitrate or nitrite, to molecular nitrogen through a series of intermediate gaseous nitrogen oxide products (Tiedje 1988). Denitrification is also commonly used to remove excessive agricultural or residential nitrogen from sewage and municipal wastewater (Rout et al. 2021, Wu et al. 2021). Denitrifiers are present among archaea and fungi, but are in the majority of cases bacteria notably the genus *Pseudomonas* (Hallin et al. 2018, Philippot et al. 2007). Denitrifiers that are present and active in the aquatic environment are confronted to human pollution, among which antibiotics and antibiotic resistance genes (ARG) considered emerging environmental contaminants notably in aquatic environments (Pruden et al. 2006).

Concentrations of antibiotics in the aquatic environment can reach up to 0.1 -2 ng L<sup>-1</sup> in surface waters (Hirsch et al. 1999, Kolpin et al. 2002, Kummerer 2009) and several hundred ng kg<sup>-1</sup> in aquatic sediments (Maghsodian et al. 2022, Zuccato et al. 2005). These environmental, also called sub-therapeutic, concentrations of antibiotics have led to the emergence of antibiotic resistant bacteria (ARB) both in the medical and natural environments (Berendonk et al. 2015). Even though antimicrobial resistance is ancient, an increase of ARB and accumulation of ARGs related to the presence of antibiotics in the environment has been confirmed (Hu et al. 2017, Pruden et al. 2006)).

Antibiotic resistance of microbes represents a threat to emerging infectious diseases and mainly the inability to treat common illnesses (Tanwar et al. 2014). The widespread presence of ARGs could contribute to the environmental resistome (Goulas et al. 2020), and possible feedback and transfer to pathogens (Baquero et al. 2008, Berendonk et al. 2015). When possessing ARG, bacteria can transfer or act as reservoirs of antibiotic resistance (Forsberg et al. 2012, Sommer & Dantas 2011). A range of ARGs are found on mobile genetic elements like plasmids. This type of easily transposable resistance has even been observed in pristine environments without any selective force of antimicrobial compounds, suggesting intrinsic resistance (Gardner et al. 1969). ARGs can thus be expected among non-pathogenic, environmental relevant microorganisms and serve as a reservoir and dissemination of ARG.

Antibiotic resistance among non-pathogens or non-clinically relevant microbes may be due to intrinsic or natural resistance against naturally produced antibiotics under environmental concentrations (Andersson & Hughes 2014). These mechanisms have existed for millions of

years and include cell wall impermeability, multidrug efflux pumps or inactivation of antimicrobial molecules or modification of the target (reviewed by (Du et al. 2018, Poole 2002a)). The microbial cell envelope may be a first barrier to certain antibiotics like macrolides (Poole 2002a, Vaara 1993a) especially in Gram-negative bacteria due to their lipopolysaccharide and outer membrane regulated by porins (reviewed by (Nikaido 2003)). In addition to this, efflux pumps are a major mechanism conferring intrinsic resistance to multiple families of antibiotics (Levy &McMurry 1978). Efflux is considered the fastest acting and most effective resistance mechanism, with an early origin (Du et al. 2018).

Antibiotic resistance and exposure to antibiotics in pathogens, from a medical point of view, is studied extensively (Amarasiri et al. 2020). Even though antibiotic resistance is ancient and omnipresent, the effect of human induced and synthetic antibiotics on environmental, non-pathogenic bacteria has received far less attention. An increasing number of studies have investigated the effect of antibiotics on biogeochemical processes and microbial communities in the environment, including denitrification (Roose-Amsaleg &Laverman 2016). However, little is known about the effect of antibiotics on the growth and activity of environmentally relevant microbes, such as denitrifiers.

In order to understand the effect of antibiotics on environmental bacteria, we assessed the antibiotic resistance of a denitrifying species isolated from a river sediment. More specifically, the effects of different antibiotics on the growth and denitrifying activity were determined. To this end, a denitrifying gram-negative bacterium was isolated in the absence of antibiotics under denitrifying conditions (nitrate, carbon, anoxia), this denitrifier was able to operate denitrification in its totality from nitrate to dinitrogen. The denitrifier, identified as *Pseudomonas veronii*, was tested towards 6 different antibiotics, covering different families and mode of actions. As a gram-negative bacterium, and due to its outer membrane characteristics, it was expected that this species would be resistant towards to macrolides such as erythromycin (ERY) and tylosin (TYL). In addition to macrolides, tetracycline (TC) and three quinolones, flumequine (FLU), ciprofloxacin (CIP) and ofloxacin (OFL) were tested. In addition to cell wall properties, efflux pumps may allow intrinsic resistance, therefore their role was assessed by using an efflux pump inhibitor while *P. veronii* was exposed to the antibiotics (Kinana et al. 2016, Reading et al. 2020). The antibiotic resistance was established by determining the minimal inhibiting concentrations, by a standard broth microdilution susceptibility method (Reller et al. 2009). In addition to this, the effect of these antibiotics on denitrifying growth and activity were investigated. Furthermore, the genome of the denitrifier

was sequenced and examined regarding antibiotic resistance genes (ARGs) using the antibiotic resistance database (CARD).

## **2. Material and Methods**

### **2.1. Denitrifier isolation and characterization**

A denitrifying bacterial strain was isolated from sediments of the Charmoise River (France), which is a tributary of the Seine River. For the isolation of the denitrifier, five grams of sediment were mixed with 50 mL sterile saline solution (2% NaCl, 0.3% MgCl<sub>2</sub>), sonicated 10 minutes (Bioblock Scientific ultrasonic cleaner 86483), shaken 20 minutes at 125 min<sup>-1</sup> and kept during 24 hours at 4°C. The resulting supernatant was diluted 100-fold and added to a denitrifying medium (pH 7, composition see Table S1) in Hungate tubes (Laverman et al. 2006). A 10-fold dilution in denitrifying medium up to 10<sup>-8</sup> was applied to the denitrifying medium containing the diluted sediment supernatant. The tubes were incubated in the dark at 25°C during 5 days to enrich denitrifiers. Denitrifying activity was tested positive by N<sub>2</sub> production in an inverted Durham-bell. The enriched denitrifiers from the highest dilution were transferred to fresh liquid medium, upon growth the enrichment was applied to solid medium (denitrifying medium with agar). Individual colonies were retested in denitrifying medium for N<sub>2</sub> production, this procedure was repeated 3 times.

Acetylene block experiments were designed to confirm the denitrification ability of this strain. The experiment had two treatments, consisted of two levels (acetylene+, where 10% of the headspace was composed of acetylene, and acetylene-, where 100% of the headspace was composed of N<sub>2</sub>). Bacteria cultures were grown under denitrifying conditions in 250 mL serum bottles sealed with rubber stoppers using a denitrifying mineral medium (Laverman et al. 2006). The 250 mL serum bottles contained 90 mL of denitrifying mineral medium. Upon sterilization the headspace was vacuumed and purged with nitrogen gas for 5 cycles to remove oxygen. The bottles were inoculated anaerobically with 10 mL bacterial culture (with a OD<sub>600</sub> of 1) to obtain a final OD<sub>600</sub> of 0.1, corresponding to approximately 10<sup>8</sup> cells mL<sup>-1</sup>. Finally, the serum bottles were cultured in an incubator with 180 rpm rotary at 28 ± 0.5 °C in dark. Samples were collected from each serum bottle by sterilized syringe 2 times every day. All groups were done in triplicates.

In addition to growth on the denitrifying medium, the isolated denitrifier was able to grow on

the Luria-Bertani (LB), Tryptic Soy Broth (TSB) and Mueller Hinton (MH) medium. The LB medium was used to test the effect of antibiotics on the growth of the isolate under optimal, aerobic conditions. The denitrifying medium was used to determine the effect of the antibiotics on denitrifying activity. Growth and cell numbers of the isolate were monitored on a regular basis at OD<sub>600</sub>. A conversion of OD<sub>600</sub> to cell numbers mL<sup>-1</sup> was based on cell counts obtained using flow cytometer counting over a range of OD<sub>600</sub>. To this end 0.2 mL of bacterial culture at different growth stages, i.e. OD<sub>600</sub> values, were diluted with filter-sterilized 5% LB and 0.15 M NaCl, in order to reach a final density of approximately 10<sup>6</sup> cell mL<sup>-1</sup>. The diluted cells (1.0 mL) were stained at room temperature together with 1 µL of SYTO® BC stain (Invitrogen™ SYTO™ Bacteria Counting Kit) for 5 minutes. The stained cells were subsequently mixed with 10 µL of microsphere standard suspension (Invitrogen™ SYTO™ Bacteria Counting Kit), and analyzed by flow cytometry (BD Accuri™ C6 Plus Flow Cytometer).

## **2.2. Genome sequencing and annotation**

The denitrifying isolate was grown in LB medium and the genomic DNA was extracted using the blood and cell culture DNA kit (Qiagen). The Spectrostar nano (BMG Labtech) was used to measure the concentration of genomic DNA and check their spectral curves (OD 230, 260, 280 nm). The genome sequence was determined using the PacBio RS II sequencing platform (Pacific Biosciences) by Eurofins genomics (SMRTbell library for sequencing on PacBio RSII) using the hair-pin adaptor protocol for continuous long read sequencing; standard insert size selection of approximately 10 Kbp for long-read protocol and sequencing with up to 10 Kbp read length (P6/C4). Annotation of the genome was performed automatically and manually using the Microscope platform ([www.genoscope.cns.fr/agc/microscope](http://www.genoscope.cns.fr/agc/microscope)) (Vallenet et al. 2017), notably offering the comparison by BLAST to the comprehensive antibiotic resistance database (CARD). Taxonomic classification was defined by topology and average nucleotide identity (ANI) based on BLAST allowing the assignation of this isolate as *Pseudomonas veronii*. The genome of *P. veronii* 11V444 was deposited in the DDBJ/ENA/GenBank European Nucleotide Archive under the accession numbers CAJCJO010000000.

## **2.3. Antibiotics and chemicals**

Six antibiotics with different modes of action were selected (Table 1). From the family of macrolides, erythromycin (ERY) and tylosin (TYL) were tested. This family of antibiotics inhibit bacterial protein synthesis (Mao &Wiegand 1968, Vazquez-Laslop &Mankin 2018) by binding to the P site reversibly on the 50S subunit of the bacterial ribosome. It has a broad spectrum

of activity against Gram-positive organisms but limited range towards Gram-negative organisms (Leclercq & Courvalin 2002). Tetracycline (TC) reversibly inhibits bacterial protein synthesis of bacteria: it binds to the 30S and 50S subunit of microbial ribosomes (Lin et al. 2018). Quinolones, ciprofloxacin (CIP), ofloxacin (OFL) and flumequine (FLU) function by inhibiting type II topoisomerase DNA gyrase, and topoisomerase IV, which is necessary to separate DNA strands, thereby inhibiting cell division (Bearden & Danziger 2001). All ATBs, as well as the efflux pump inhibitor, were purchased from Sigma-Aldrich.

Table 1. The tested antibiotics, their families, abbreviations and modes of action.

Family	ATB	Concentration range (mg L <sup>-1</sup> )	Mode of Action:
Macrolide	Erythromycin	1000, 750, 375, 188, 94, 47	protein synthesis
	Tylosin	2500, 1000, 500, 250, 125, 62.5	
Tetracycline	Tetracycline	16, 8, 4, 2, 1, 0.5	
Quinolone	Ciprofloxacin	2, 1, 0.5, 0.25, 0.125, 0.0625	DNA gyrase/topoisomerase
	Ofloxacin	2, 1, 0.5, 0.25, 0.125, 0.0625	
	Flumequine	16, 8, 4, 2, 1, 0.5	

## 2.4. Dilution Susceptibility Method (DSM)

The dilution susceptibility approach was performed using a range of 7 concentrations for each antibiotic (see Table 1) in 96-well plates in LB medium. A preculture of the strain was grown on LB medium overnight at 25°C stirring 150 rpm. The precultured bacteria were diluted with LB medium to a final OD<sub>600</sub> of 0.2. Experimental wells containing 100 µL of diluted bacteria culture, 50 µL of LB and 50 µL of antibiotic solution with a final OD<sub>600</sub> of 0.1, corresponding to approximately 10<sup>8</sup> cells mL<sup>-1</sup> were exposed in triplicate to each antibiotic. The antibiotic solution was replaced by 50 µL sterile water in the positive control. A well containing 150 µL of LB and 50 µL of ultra-pure water was used as a negative control. The plates were incubated 24 h at 25 °C in the dark in the microplate reader (Spectrofluorimeter Xenius XM, SAFAS), continuously orbital shaken (4 Hz), measuring the OD<sub>600</sub> every 10 minutes.

The efflux pump inhibitor (EPI) Phe-Arg β-naphthylamide (PAβN) dihydrochloride (CAS Number: 100929-99-5) was added at 125 mg L<sup>-1</sup> final concentration according to (Reading et al. 2020).

The minimum inhibitory concentration (MIC<sub>80</sub>), the lowest concentration (mg/L) of antibiotic at which growth is inhibited by 80% was determined by plotting OD<sub>600</sub> to ATB concentrations.

## 2.5. Impact of antibiotics on denitrifying growth and activity

Based on the DSM tests, concentrations of the six antibiotics were selected that were below

the MIC<sub>80</sub> value to determine a possible effect on denitrifying growth and activity. To this end *P. veronii* was grown under denitrifying conditions in 120 mL serum bottles sealed with rubber stoppers using a denitrifying mineral medium (Laverman et al. 2006). Growth was determined by OD<sub>600</sub> and denitrifying activity was monitored as the disappearance of nitrate, the production and reduction of nitrite over time. The 120 mL serum bottles contained 90 mL of denitrifying mineral medium, similar to that used for the isolation of *P. veronii*. Upon sterilization the headspace was vacuumed and purged with nitrogen gas for 5 cycles to remove oxygen. The bottles were inoculated anaerobically with 10 mL bacterial culture (with a OD<sub>600</sub> of 1) to obtain a final OD<sub>600</sub> of 0.1, corresponding to approximately 10<sup>8</sup> cells mL<sup>-1</sup>. The six antibiotics were added into the mineral medium and the final concentrations were set as follows: ERY 100 mg L<sup>-1</sup>, TYL 100 mg L<sup>-1</sup>, TC 0.5 mg L<sup>-1</sup>, CIP 0.25 mg L<sup>-1</sup>, OFL 0.25 mg L<sup>-1</sup>, FLU 8 mg L<sup>-1</sup>. Finally, the serum bottles were cultured in an incubator with 180 rpm rotary at 28 ± 0.5 °C in dark. Samples were collected from each serum bottle by sterilized syringe 2 times every day for a total time of 4 days. All groups were done in triplicates.

## **2.6. Data processing and EC<sub>50</sub> calculation**

The Kruskal-Wallis non parametrical test was used to identify significant effects of antibiotics on the growth of the *Pseudomonas veronii* via Sigmaplot 12.0.

# **3. Results and discussion**

## **3.1. Identification and growth of the denitrifying isolate**

The taxonomic classification showed that the isolated denitrifier can be identified as *Pseudomonas veronii*. This Gram-negative bacterium was first isolated from a French natural mineral water source (Elomari et al. 1996). No clinical significance has been found so far indicating that *P. veronii* is a pathogen and it has been studied only occasionally. It belongs to the *P. fluorescens* sub group (among 8 sub-groups) of the *P. fluorescens* lineage among the 3 known lineages of the genus (Lalucat et al. 2020, Silby et al. 2011).

*P. veronii* growth on LB medium shows a maximum growth rate after 6 hours with a doubling time of approximately 1.5h (+/-) and cell numbers 4.3 \* 10<sup>8</sup> cell mL<sup>-1</sup> (see Figure S1). No differences were observed on growth rate and cell numbers between growth on LB and TSB medium (see Figure S2). Acetylene block experiments showed that NO<sub>2</sub><sup>-</sup> is further transformed

to N<sub>2</sub>O (see Figure S3).

### 3.2. ARG analysis

Submitted to CARD, the genome of *P. veronii* was revealed to contain four antibiotic resistance genes corresponding to gene encoding efflux pumps. Searching directly efflux pump genes by keywords in the genome of *P. veronii*, 9 additional genes encoding efflux pumps were identified. Among the 13 genes, most of them (10/13) were from the Resistance-Nodulation-Division superfamily (RND), which are pumps specific for Gram-negative bacteria. *P. veronii* genome harbors: three genes encoding *acrA*, three *acrB*, one *mdtA*, one *mdtB*, one *mdtC* and one *ttgT* from RND and one *emrA*, one *emrB* from major facilitator superfamily (MFS) and one *SugE* from small multidrug family.

At least two genes encoding efflux pump regulators were found: *soxR* and a putative MER family regulatory protein.

Porin genes were also checked in the genome of *P. veronii* as they are a major component of gram-negative outer membrane regulating the crossing of molecules through it. Thirty-one porin and porin-like genes were detected (among them, 15 were from *Opr* family, 2 *gal* and 5 *nicP*).

### 3.3. Growth of *P. veronii* in the presence of antibiotics

#### 3.3.1. Dilution Susceptibility and disc tests

The growth of *P. veronii* in the presence of the six tested antibiotics are shown in Figure 1 presenting 3 out of the 7 tested concentrations. *P. veronii* grows on LB reaching maximum cell numbers after 12 hours. In the presence of ERY, even at the highest concentrations, little impact on growth is observed. A maximum inhibition on growth reaches 15% at the highest applied concentration of 1,000 mg L<sup>-1</sup>. The cell numbers of *P. veronii* decrease with increasing concentrations of TYL with a decrease up to 60% at 2,500 mg L<sup>-1</sup> and around 30% inhibition at 250 mg L<sup>-1</sup> L. In the presence of TC, growth is slightly reduced at a concentration of 0.5 mg L<sup>-1</sup> (30%) and up to 70% at 4 mg L<sup>-1</sup> and higher.

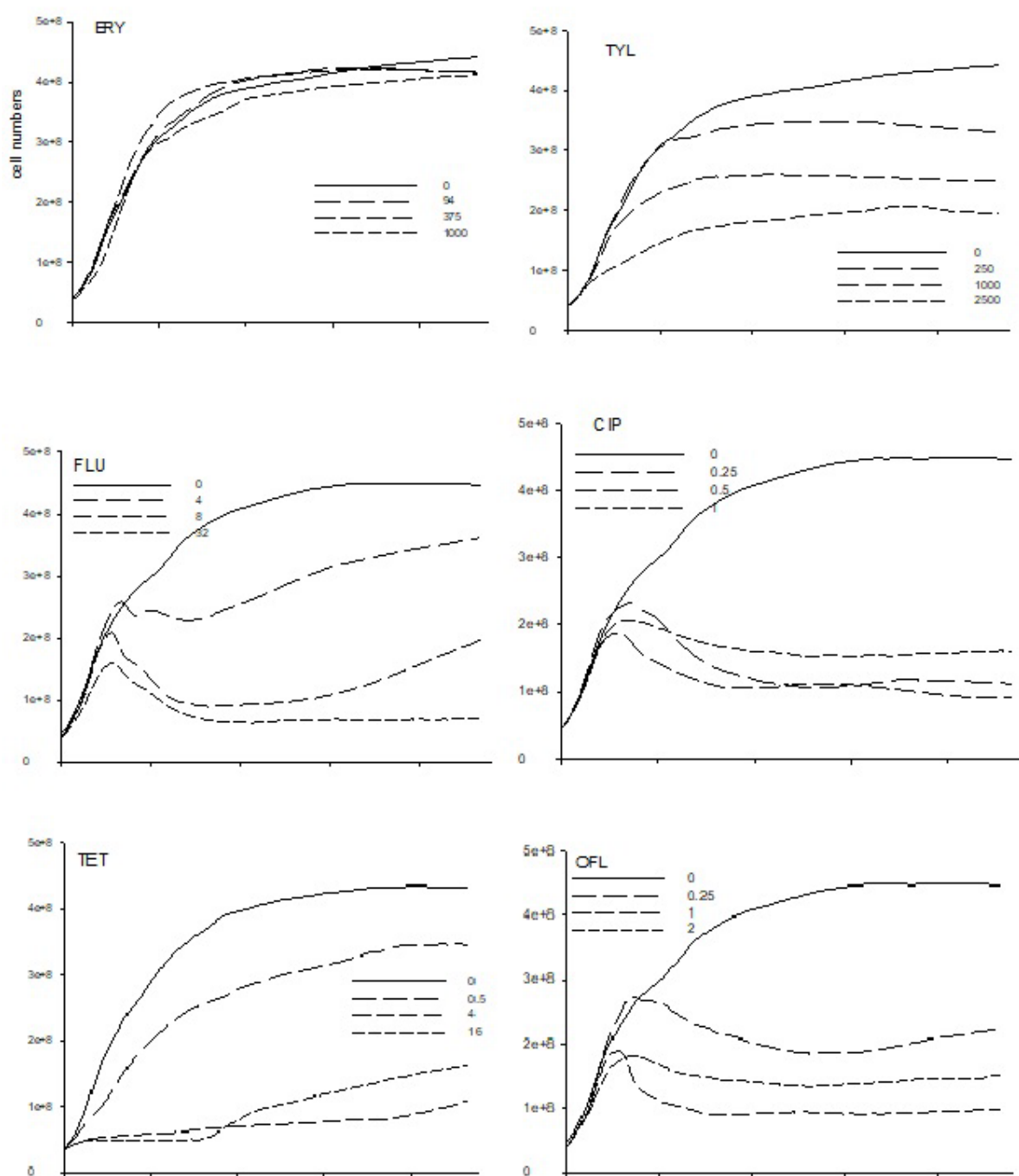


Figure 1. Growth curves of *P. veronii* in the presence of the antibiotics ERY, TYL, TC(TET), FLU, CIP and OFL at 3 different concentrations, an overview of the results for all tested concentrations are presented as Figure S4.

In the presence of the fluoroquinolons CIP, FLU and OFL growth starts similar to the control for approximately 2 hours, after which cell numbers decrease. The decrease in cell numbers varies between 15 to 70% with increasing FLU concentrations (4 to  $16 \text{ mg L}^{-1}$ ). At OFL concentrations of  $0.25 \text{ mg L}^{-1}$  ~50% inhibition and at 1 and 2 between 80% and 85% is observed. Variations

are observed between the different tests, whereas the triplicates results were always within 10%. The highest inhibitions in cell numbers are observed in the presence of CIP, at concentrations of 0.5 mg L<sup>-1</sup> this inhibition is around 70%, with 50-90% at 1 and 2 mg L<sup>-1</sup>.

In addition to growth at different concentrations, *P. veronii* was grown on LB agar plates using the disc diffusion method, results of the data are listed in Table 2. The disc diffusion tests showed a resistance of *P. veronii* towards ERY, TYL and FLU, and a susceptibility towards TC, OFL and CIP.

Based on the growth curves towards the tested antibiotics (see Figures S4), the response of *P. veronii* was determined to obtain MIC<sub>80</sub> values for the different antibiotics and is summarized in Table 2. The MIC<sub>80</sub> is defined as the lowest concentration that inhibits ≥80 % of growth as compared to the non-exposed control (EUCAST 2021). Based on the values for clinical species the different antibiotics regarding and the designation of susceptible (S), susceptible at increased exposure (I) and resistant (R) (EUCAST 2019) *P. veronii* are defined according to this.

Table 2. Resistance of *Pseudomonas veronii* towards the different tested antibiotics, expressed as the Minimum Inhibition Concentration (MIC<sub>80</sub>: lowest concentration inhibiting ≥ 80 % growth compared to control) together with the disc results (Figure S5). S indicates susceptibility, I susceptible at highest exposure and R being resistant, in grey the reference MIC values for each antibiotic.

Family	ATB	[Max ] mg L <sup>-1</sup>	MIC <sub>80</sub> mg L <sup>-1</sup>	S/R (I)	S</R>* mg L <sup>-1</sup>	disc
Macrolide	ERY	1,000	>1,000	R	1/4 <sup>b</sup>	R
	TYL	2,500	>2,500	R	4/16 <sup>a</sup>	R
Tetracycline	TC	16	>16	R	4/8 <sup>b</sup>	S
Quinolone	FLU	32	16	R	4/8 <sup>b</sup>	R
	OFL	4	1	I	1/4 <sup>b</sup>	I
	CIP	2	0.25	S	1/2 <sup>b</sup>	I

<sup>a</sup>, Values based on the “Clinical and Laboratory Standards Institute” (CLSI or

<sup>b</sup>, the “Comité de l'Antibiogramme de la Société Française de Microbiologie” (CA-SFM).

*P. veronii* shows high MIC<sub>80</sub> values exceeding the maximum tested concentrations towards the macrolides ERY and TYL (> 1,000 mg L<sup>-1</sup> and 2,500 mg L<sup>-1</sup>), and TC (> 16 mg L<sup>-1</sup>). *P. veronii* is considered resistant (R) to all these compounds based on the values defined by the CLSI and CA-SFM. *P. veronii* had lower MIC toward quinolones (OFL and CIP): 1 and 0.25 mg L<sup>-1</sup>, respectively. *P. veronii* could be described as susceptible at highest exposure (I) towards OFL and susceptible (S) towards CIP. The addition of the efflux pump inhibitor decreased the MICs of OFL to 0.25 mg L<sup>-1</sup>, for CIP this value remained 0.25 mg L<sup>-1</sup>; *P. veronii* became susceptible to the 2 antibiotics.

The objective of this part was to estimate the MICs (mg L<sup>-1</sup>) of *P. veronii* against antibiotics via dilution susceptibility and disc diffusion tests under medical based standards. Results showed that, on one hand, *P. veronii* was highly tolerant (*i.e.* able to grow at all tested concentrations) to macrolides (ERY, TYL), tetracycline (TC), and one quinolone (FLU). However, *P. veronii* was sensitive towards the quinolones ciprofloxacin (CIP) and ofloxacin (OFL).

*Pseudomonas* represents a ubiquitous genus among Gram-negative bacteria showing a high diversity (Peix et al. 2018, Silby et al. 2011), notably due to its metabolic versatility. Antibiotic resistance has been described extensively in the pathogen *Pseudomonas aeruginosa* (e.g. (Botelho et al. 2019, Breidenstein et al. 2011, Poole 2014)) whereas recently also in other *Pseudomonas* species isolated from the environment (Camiade et al. 2020).

As hypothesized, *P. veronii* was resistant towards the macrolide erythromycin and tylosin, most likely due to its outer membrane exclusionary properties. *P. veronii* as a gram-negative bacterium presents a tripartite cell envelope consisting of three layers: the inner phospholipid or cytoplasmic membrane, the peptidoglycan cell wall, and the outer lipopolysaccharide membrane (reviewed by (Alav et al. 2021)). Due to their structure, these lipid bilayers show little permeability to hydrophilic compounds. Furthermore, *Pseudomonas aeruginosa* is characterized by a very low permeability of its outer membrane, representing about 8% that of *E. coli* (Chevalier et al. 2017, Hancock & Brinkman 2002). As part of the *Pseudomonas* family, *P. veronii* could have similar low permeability that reinforce their resistance towards macrolides (Leclercq & Courvalin 1991, Vaara 1993b).

The bacterial outer membrane contains channel-forming proteins; porins allowing passage of molecules (reviewed in (Nikaido 2003)). According to Nikaido (2003), small antibacterial agents such as tetracycline and fluoroquinolones use porin channels for penetration, at least in the Enterobacteriaceae. The presence of porins and diffusion would explain the lower resistance of *P. veronii* towards FLU and its susceptibility towards CIP and OFL. Genome analysis showed the presence of several porins, confirming the importance of these proteins in the susceptibility of bacteria towards certain antibiotics.

### **3.3.2. Resistance of *P. veronii* related to efflux pumps**

The growth of *P. veronii* was tested in the presence of the different antibiotics and the efflux pump inhibitor (EPI) in order to establish the role of efflux pumps in the antibiotic resistance. In Figure 2 the inhibition at high and low concentrations of the six tested antibiotics is shown in the presence and absence of EPI. At the high antibiotic concentrations, the addition of EPI

showed significant higher susceptibility of *P. veronii* in the presence of ERY (t test  $p < 0.005$ ). Even though a slight increase in susceptibility in the presence of EPI is observed for TYL, TC, FLU, OFL and CIP, these differences were not significant (t-test  $p > .1$ ). At low concentrations, the efflux pumps were more important; *P. veronii* was less resistant towards CIP, OFL, FLU and TYL in the presence of the efflux pump inhibitor. No effect was observed at low concentrations of ERY and only a minor effect was observed in the presence of TC.

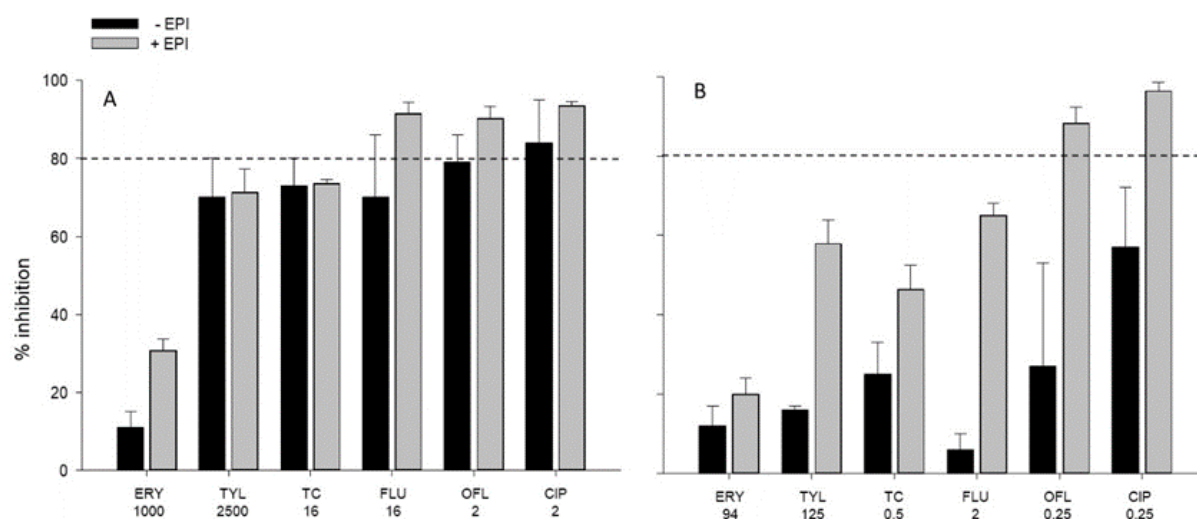


Figure 2. Growth inhibition of *P. veronii* at high (A) and low (B) antibiotic concentrations, in the absence and presence of the efflux inhibitor EPI.

*P. veronii* remained resistant to most of the antibiotic compounds in the presence of the efflux pump inhibitor, however in most of the cases the MIC decreased. A 4-fold decrease for FLU and OFL. The efflux pump inhibitor showed little change in MIC for TC.

The AcrAB-TolC efflux pump present in the *P. veronii* genome is an intrinsic RND efflux pump first found in *Escherichia coli* and widely conserved throughout Gram-negative organisms (Poole et al. 1993). The percentage of similarity of the 3 detected AcrB genes in *P. veronii* were 97% /99% /95% compared with *Pseudomonas extremaustralis*. The AcrAB-TolC efflux pump, as well as other RND family members, forms a tripartite assembly structure with its partner protein, which makes them easier to efflux ATBs out of the cell by binding substrates at the outer leaflet of the inner membrane and periplasm (Du et al. 2018). The AcrAB-TolC in *E. Coli* has been shown to provide resistance towards the ERY and TC (Ma et al. 1993, Poole 2002b). The role of this efflux pump for ERY complements the role of the cell wall/porins as described earlier. This is confirmed by the effect of the efflux inhibitor, resulting in a reduced resistance towards ERY. Resistance towards quinolones could be due to an overexpression of efflux pumps.

*P. veronii* seems not in the possession of any acquired resistance genes, in agreement with its susceptibility towards CIP and OFL. The incomplete inhibition of growth might very well be due to efflux activity. In the presence of the efflux inhibitor, *P. veronii* is more susceptible towards FLU and OFL (>80%). On the contrary in the presence of EPI and CIP *P. veronii* is more resistant.

Even though it could have been expected that AcrAB might provide resistance towards TC, the presence of the efflux pump inhibitor is minor. This suggests that efflux pumps are not involved in the resistance towards TC. Possible explanations based on the chemical properties of TC are proposed (Fuoco 2012): 1) the linearly arranged naphthacene ring system of TC may not suit for AcrAB system; 2) the lipophilicity of TC could enhance its biological potency and dynamics; 3) the radical oxygen species produced by TC may already bring irreversibly damages to macromolecules like RNA and proteins in bacteria.

### 3.4. Growth and activity of *P. veronii* under denitrifying conditions

The growth and activity of *P. veronii* was tested under denitrifying conditions for the different antibiotics. Growth of *P. veronii* under denitrifying conditions compared to growth in LB medium showed similar trends. The results of nitrate reduction and intermediate nitrite production in the presence of TYL, ERY, TC, CIP, OFL and FLU are shown in Figure 3B (in mg L<sup>-1</sup>).

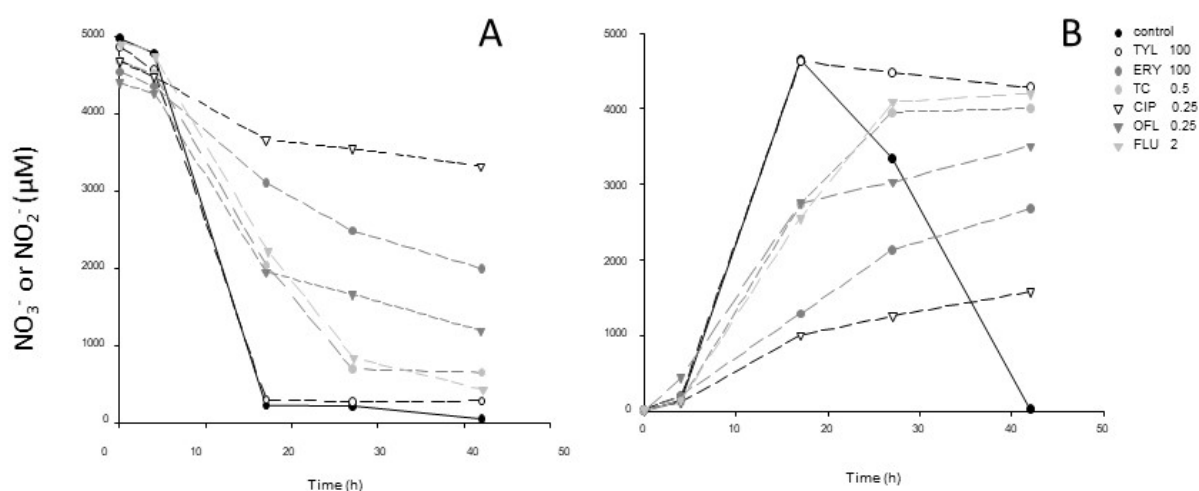


Figure 3. Changes in nitrate (A) and nitrite (B) concentrations over time in cultures of *P. veronii* on denitrifying medium in the presence of the 6 tested antibiotics

Growth of *P. veronii* under anoxic conditions in the mineral medium is accompanied by the reduction of NO<sub>3</sub><sup>-</sup> and the transient production of NO<sub>2</sub><sup>-</sup> (see Figure 3). Exposure to TYL and TC slightly inhibited growth under denitrifying conditions (less than 10%, see Table 3). ERY, FLU and OFL inhibited growth with respectively 17, 21 and 37% with the highest impact of CIP (55%

inhibition). The changes in nitrate and nitrite concentrations in the presence of the antibiotics (Figure 3), show the effect of the six antibiotics on the denitrifying activity of *P. veronii*. The control, without antibiotics shows a rapid decrease in nitrate concentrations with an increase and consequent decrease of intermediate nitrite, further reduced to N<sub>2</sub>O and N<sub>2</sub>. In the presence of TYL, with little impact on the growth of *P. veronii*, nitrate is reduced at a similar rate as the control, however nitrite reduction is stalled. The presence of the ERY, FLU, OFL, TC and CIP slow down nitrate reduction and all result in the accumulation of nitrite.

Table 3. Effect of the exposure to the six antibiotics on growth of *P. veronii* under denitrifying conditions, the growth inhibition compared to the unamended control, the nitrate reduction and nitrite accumulation rates.

Treatment	Growth inhibition	Nitrate reduction rate ( $\mu\text{mol cell h}^{-1}$ )	Nitrite accumulation ( $\mu\text{mol cell h}^{-1}$ )
control		364	1.1
TYL 100	<10%	345	349
ERY 100	21%	132	138
TC 0.5	<10%	154	153
CIP 0.25	55%	99	112
OFL 0.25	37%	158	172
FLU 2	17%	193	182

Taking into consideration the decrease in cell numbers, i.e. growth, nitrate reduction rates were calculated per cell number (Table 3) highest rates were determined in the control and in the presence of TYL, which indicates that TYL at the tested concentration do not affect the nitrate reduction process of *P. veronii*. The nitrate reduction rates normalized per cell undergo the strongest effect, with lowest nitrate reduction rates in the presence of CIP, whereas ERY, TC and OFL share approximately the same inhibition effect. The nitrate reduction rates were thus affected and resulted in a proportional production of nitrite that appears to be not further reduced to gaseous nitrogen.

The nitrate reductase (NAR) in *P. veronii* is responsible for the first step, reducing nitrate to nitrite, while the nitrite reductase (NIR) is responsible for the second step (i.e. reducing nitrite to NO and N<sub>2</sub>O). Previous studies have reported that different antibiotic family at varying degrees could have adverse or inhibitory impacts on denitrification, mainly due to their effects on denitrifying enzymes or genes (Chen et al. 2017, Guo et al. 2020, Li et al. 2021, Su et al. 2019, Zhu et al. 2017). Nitrate reduction rate was significantly inhibited by a concentration of 2 mg L<sup>-1</sup> sulfamethoxazole/tetracycline, together with an 86% decrease of the relative denitrifier abundance (Zhu et al. 2017). Expression of *nirS* gene (coding NIR) was inhibited under 24 hours exposure of different antibiotics including ciprofloxacin (CIP),

sulfamethoxazole (SMX), chlortetracycline (CTC) and amoxicillin (AMX) (Li et al. 2021). This is in line with the results of the current study, showing nitrate reduction inhibition for 5 of 6 tested antibiotics (ERY, TC, CIP, OFL and FLU), leading to the accumulation of nitrite. Possibly, these antibiotics affected the expression of nirS as was shown in previous studies. Whereas some effect on growth was observed, the effect on the denitrification process, *i.e.* incomplete denitrification with accumulation of nitrite appears an additional effect of the exposure to antibiotics.

Overall, the accumulation of nitrite can have negative impacts on aquatic ecosystems and human health, as nitrite can be toxic and contribute to eutrophication. Therefore, it is important to consider the effects of antibiotics on denitrification and take measures to reduce their environmental presence.

## 4. Conclusions

*Pseudomonas veronii*, a gram negative denitrifier isolated from river sediment, appears to be either resistant or susceptible depending on the type of tested antibiotic. Based on the standardized antibiotic resistance tests, *P. veronii* was highly resistant towards the macrolide erythromycin and, albeit to a lesser extent, tylosin. *P. veronii* can be considered intermediate resistant towards tetracycline and flumequine and susceptible towards ofloxacin and ciprofloxacin. The presence of AcrAB-TolC efflux pump in *P. veronii* could explain ATBR, and overexpression of it could thus increase ATBR. Further experiments on AcrAB-TolC efflux gene transcription and protein expression are needed to see if this efflux pump is overexpressed. Despite its resistance to some of the antibiotics, the production and reduction of nitrite appears to be quite sensitive towards most of the tested antibiotics, resulting in nitrite accumulation.

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## **Supporting information**

Antimicrobial resistance in the riverine denitrifying *Pseudomonas veronii*

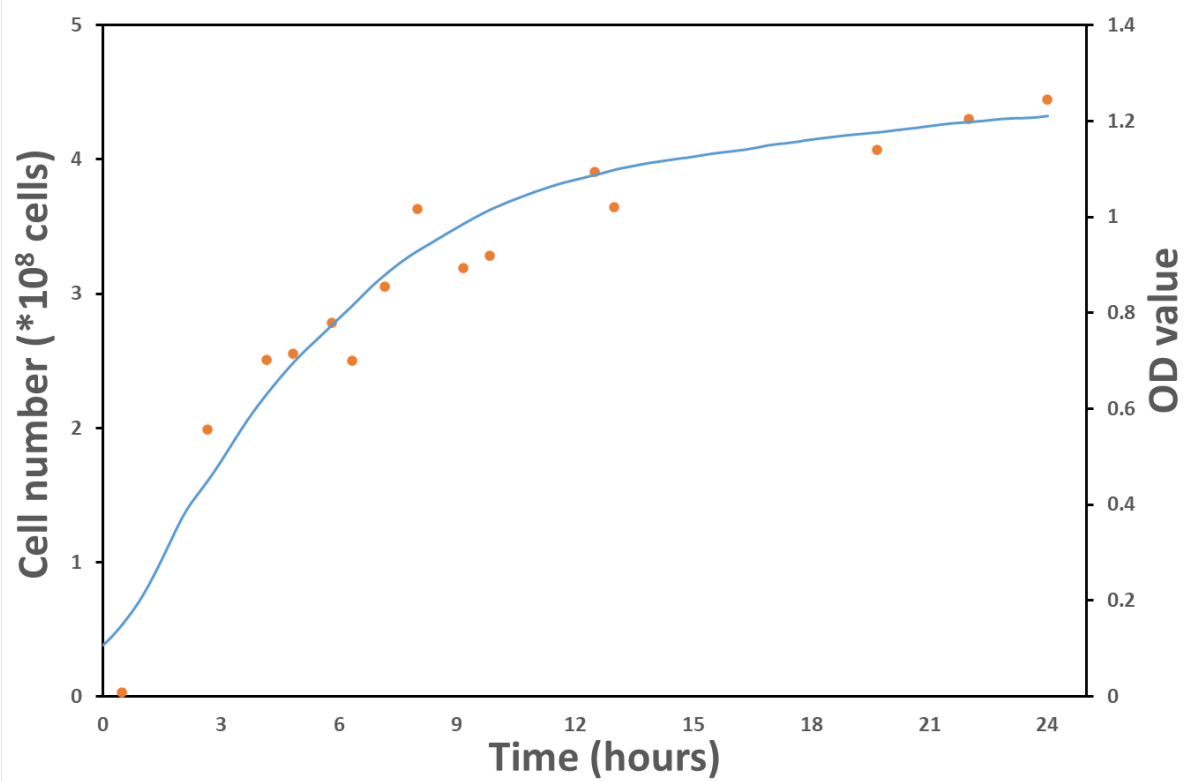


Figure S1. Growth curve of *P. veronii* on LB medium.

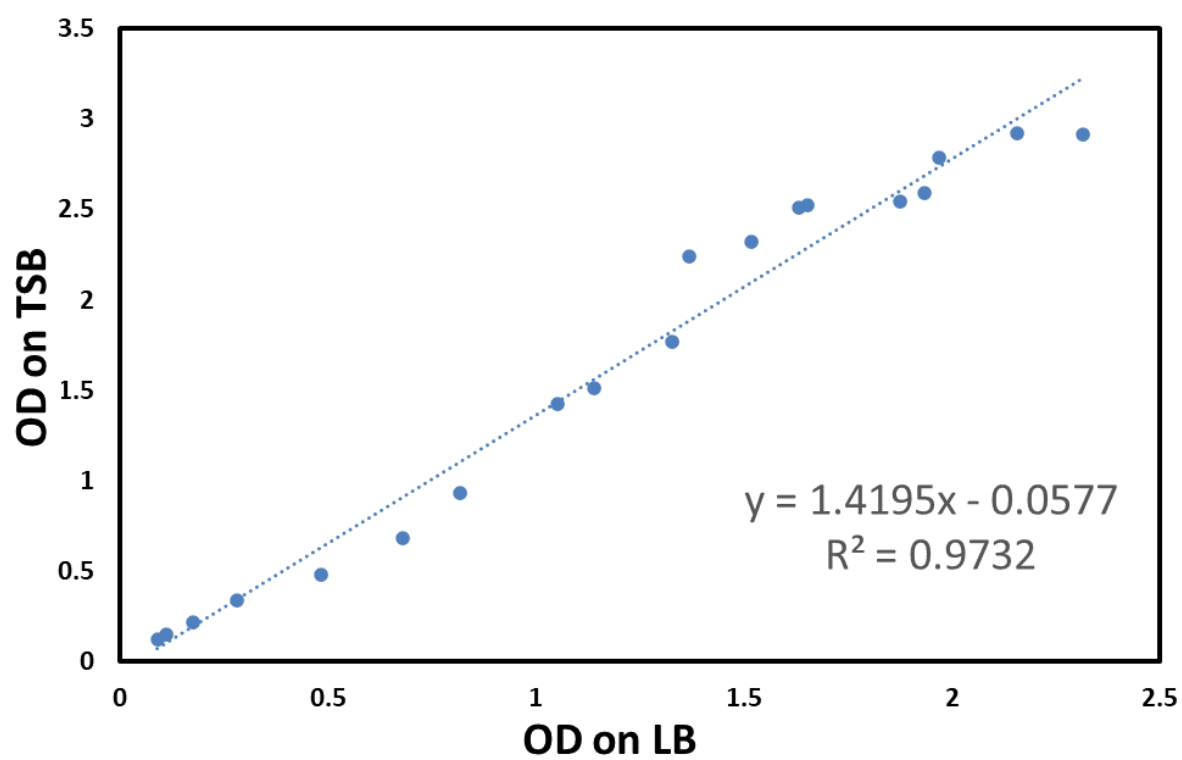


Figure S2. Comparison of bacteria growth on LB with TSB with the liner fit (via Excel 2016)

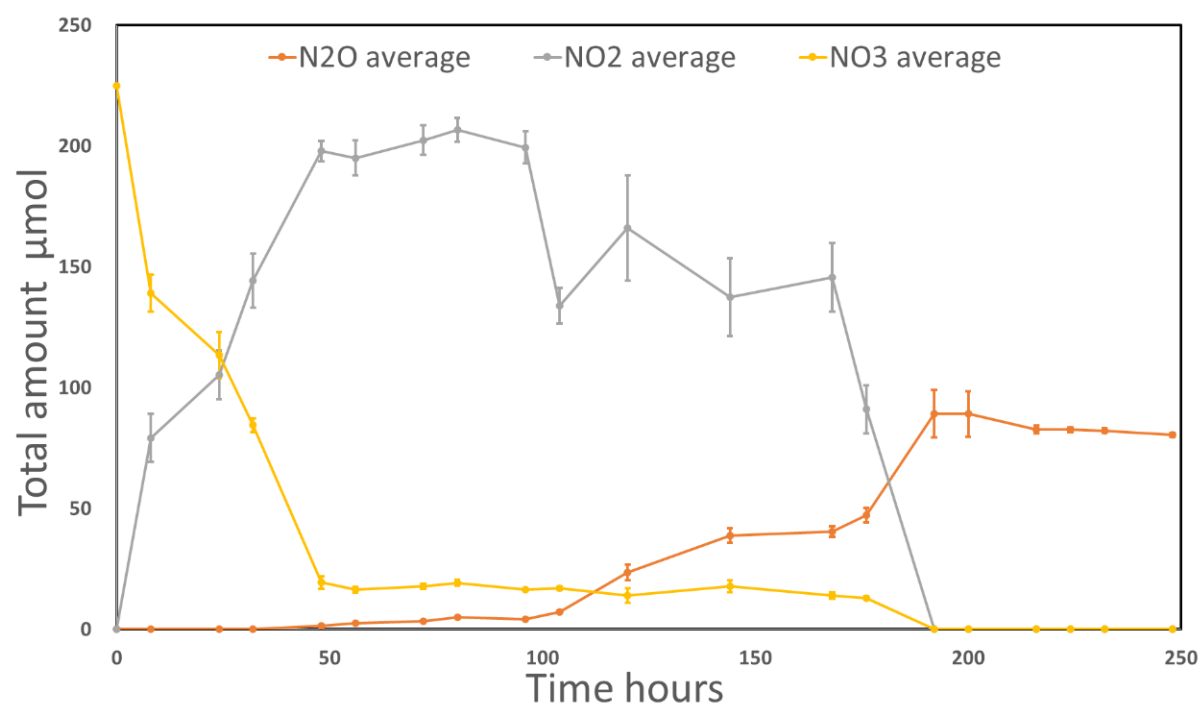


Figure S3. Acetylene block experiments of *P. veronii* on denitrification medium, showing the process of nitrate reduction, nitrite reduction and N<sub>2</sub>O production (n=3).

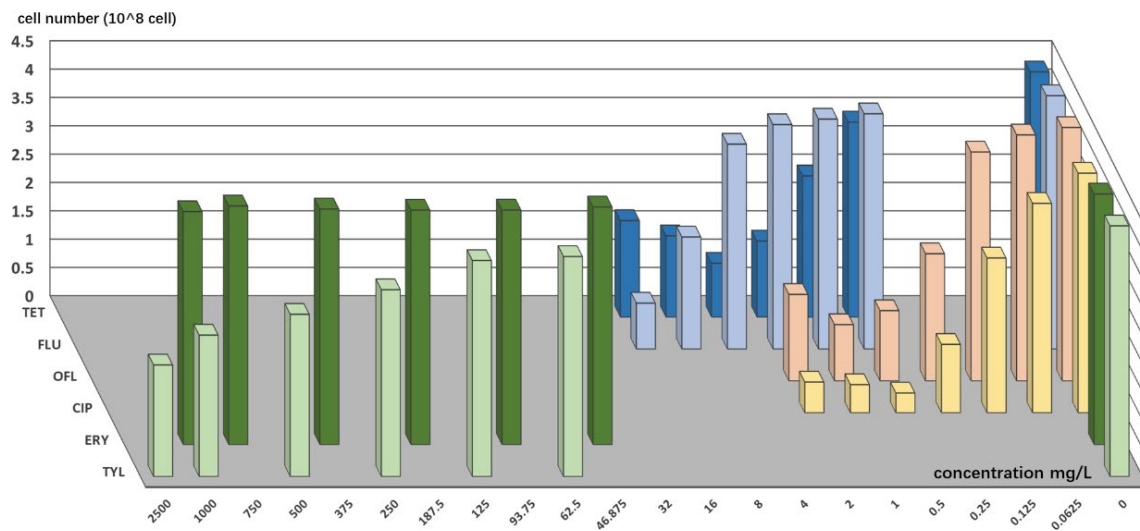


Figure S4. Overview of the cell numbers of *P. veronii* after 24 hours growth in the presence of the antibiotics ERY (dark green), TYL (light green), TC(TET, dark blue), FLU (light blue), CIP (yellow) and OFL (orange) at all tested concentrations.

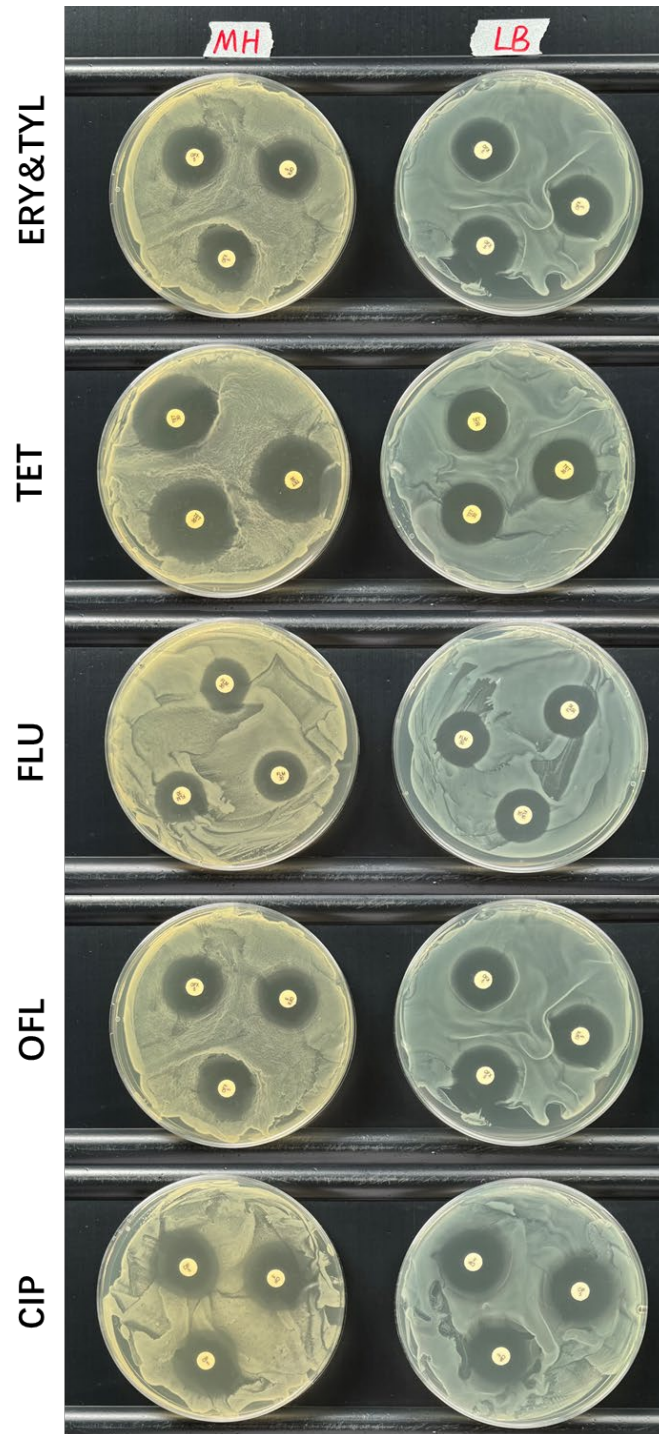


Figure S5. Overview of the disc tests of *P. veronii* after 24 hours growth in the presence of the antibiotics ERY, TYL, TC(TET), FLU, CIP and OFL under 2 different agar medium (MH/LB).

Table S1. Composition and concentration of mineral medium for denitrification determination

Recipe of classic mineral medium (500mL)		
Compound	Concentration mM	total volume mL
Solution A		
KH <sub>2</sub> PO <sub>4</sub>	1.5	50
K <sub>2</sub> HPO <sub>4</sub>	1.5	
Solution B		
NH <sub>4</sub> Cl	1	450
KCl	4	
CaCl <sub>2</sub>	1	
MgCl <sub>2</sub>	2.5	
KNO <sub>3</sub>	5	
Na-succinate	5	
Na-acetate	5	
lactate	5	
yeast extract	0.1g L <sup>-1</sup>	
trace elements	1mL L <sup>-1</sup> SL9	



## **Chapter 3**

**Mineral induced antibiotic transformation  
byproducts control on the bacteria growth  
and denitrification activity**

**In preparation**



## Abstract

Although abiotic transformations of quinolones and tetracyclines by redox-active minerals are widely investigated, little is known about the antimicrobial activity and toxicity of the transformation byproducts. In this work, we first studied the mechanisms of transformation of ciprofloxacin (CIP) and tetracycline (TC) by a common redox soil mineral, birnessite ( $\text{MnO}_2$ ), and then the effects of transformation byproducts on both growth and activity on a denitrifying isolate. At pH 5, four different byproducts of CIP and five oxidation byproducts of TC were identified after reaction with birnessite identified by liquid chromatography-mass spectrometry, and monitored over a range of reaction time (0-6 hours). For CIP, impact on the growth of denitrifier *Pseudomonas veronii* decreased over reaction time where only the first CIP byproduct ( $m/z$  334) resulting from the opening of piperazine ring kept some of its antimicrobial activity. Closed batch experiments showed that the nitrite reduction rate was affected by CIP or byproducts while nitrate reduction rates remained stable. For TC, the antimicrobial activity of TC byproducts disappeared with the reaction processing, as well as the nitrate and nitrite reduction inhibition activity. The activities of nitrite reductases appeared to be affected by CIP and TC, compared to nitrate reductases. These findings have important implications in the assessment of antibiotics effects and their abiotic transformation byproducts in the environment.

**Keywords:** antibiotic; degradation; minerals; nitrate reduction; denitrifiers.

## 1. Introduction

The extensive use of antibiotics in human and veterinary health, and their consequent presence in the environment are of increasing concern (Kümmerer 2009). The presence of antibiotics in the environment could cause potential risk in sustaining and spread of antibiotic resistant bacteria and genes as well as on ecosystem functioning (Kim & Carlson 2007). Major families of antibiotics, like fluoroquinolones and tetracyclines, are widely used in both human and veterinary medicine.

A frequently detected antibiotic in the environment is the fluoroquinolone ciprofloxacin (CIP). Concentrations of CIP in the  $\text{ng L}^{-1}$  range were detected in river water (Sodhi & Singh 2021, Tamtam et al. 2008), and in the  $\mu\text{g kg}^{-1}$  in soil and sediment (Dinh et al. 2017a, Huang et al. 2013). Ciprofloxacin concentrations in sediments have increased over the past decades due to its high adsorption capacities (Agunbiade & Moodley 2016, Gibs et al. 2013, Zhou et al. 2011). The fate of ciprofloxacin in soil and sediments has been extensively studied (Chen et al. 2022, Pavlović et al. 2017, Tan et al. 2015, Vasconcelos et al. 2009, Zhou et al. 2019). It was shown that CIP can be transformed upon interactions with environmental mineral surfaces, such as hematite and birnessite (Martin et al. 2015, Tu et al. 2014, Xiao et al. 2013, Zhang & Huang 2005, Zhou et al. 2019).

Tetracycline (TC) is another commonly detected antibiotic in the environment. It has been found in various environmental matrices including surface water, groundwater, and soils (Conde-Cid et al. 2020, Halling-Sørensen et al. 2002, Jacobsen & Berglund 1988, Kühne et al. 2000, Luo et al. 2011), up to  $\text{ng L}^{-1}$  (Dinh et al. 2017b). TC adsorption capacity in soils and sediments is relatively high compared to other antibiotics, which strongly depends on environmental factors and sediment/soil properties (Kim et al. 2005, Rabølle & Spliid 2000, Sassman & Lee 2005, Xu & Li 2010, Zhang et al. 2010). As for CIP, TC can be also chemically degraded through interactions with redox-active minerals (Chen & Huang 2011, Wang et al. 2016, Xu & Li 2010, Zhang et al. 2019).

Although the degradation of TC (Yin et al. 2020, Żyła et al. 2021) and CIP (Liao et al. 2016, Rusch et al. 2019) has been evaluated, the impacts of transformation products on microbial communities and ecosystem functioning are scarcely investigated. Possible antimicrobial activity of the CIP byproducts has only been studied on growth of pathogens like *E. coli* (Phillips et al. 1990, Sunderland et al. 2001), as well as TC byproducts on tetracycline-resistant strains (Halling-Sørensen et al. 2002). However, their impacts on environmental bacteria, particularly

beneficial microbes playing a role in the biogeochemical cycling of elements, remains rare. In the current study, the effects of mineral induced transformation byproducts on both bacterial growth and activity were investigated. The antimicrobial activity of CIP or TC and corresponding byproducts on *P. veronii* was evaluated under pure cultured conditions by a broth micro-dilution susceptibility method (Reller et al. 2009). The toxicity of CIP or TC on denitrification activity of *P. veronii* was also determined in mineral medium via bottle test. Consequently, we could give reasonable prediction on ecotoxicity of CIP or TC in manganese-containing sediment environments.

## **2. Material and Methods**

### **2.1. Chemicals**

All chemicals were analytical-reagent grade or higher purity and solvents were HPLC grade. Tetracycline and ciprofloxacin were purchased from Sigma-Aldrich. Sodium chloride and oxalic acid dihydrate were purchased from Sigma-Aldrich. Acetonitrile, ethanol and methanol were purchased from Mallinckrodt Baker. Water was purified using a Nanopure system.

### **2.2. Birnessite synthesis and characterization**

Acid birnessite was prepared following the procedure of McKenzie (McKenzie 1971). 166 mL of concentrated HCl was added dropwise to 2.5 L of 0.4M  $\text{KMnO}_4$ . The suspension was stirred vigorously and kept at 90 °C during the HCl addition. The reaction was continued at 90 °C for a further 10 min. Then, the obtained slurry was stirred vigorously at room temperature for 15 h. The precipitate was collected by centrifugation and washed with ultrapure water repeatedly until the conductivity was close to  $0.6 \mu\text{S cm}^{-1}$ . The suspensions were stored in polypropylene containers at 4 °C for further use.

The purity of acid birnessite was confirmed by powder X-ray diffraction (XRD) (Figure S1). The XRD data were recorded with monochromatized  $\text{CuK}\alpha$  radiation on a Bruker AXS D8 Advance diffractometer ( $\theta$  -  $2\theta$  Bragg-Brentano geometry) over the range of 10 - 100°  $2\theta$  at a step size of 0.02°. The  $\text{N}_2$  Brunauer-Emmett-Teller (BET) specific surface area of the synthetic acid birnessite was  $65 \pm 1 \text{ m}^2 \text{ g}^{-1}$ .

### 2.3. Birnessite induced CIP/TC byproducts

Ten ml plastic tubes (Falcon) were wrapped with aluminum foil to eliminate the possibility of a photolysis reaction. 12.5  $\mu\text{L}$  of birnessite stock solution (concentration 80 g  $\text{L}^{-1}$ ) and 330  $\mu\text{L}$  CIP stock solution (concentration 3 mM)/TC stock solution (concentration 12mM) was applied to each tube, made up to 10 mL with ultrapure water and adjust to pH 5 by NaOH/HCl solutions (Li et al. 2022). The tubes were flushed by nitrogen to avoid oxygen in the air. The tubes were then put in an auto shaker, shaking at 250 rpm under room temperature. After shaking for a certain time (0 min as control, 20, 30, 40, 50, 60, 70, 80, 90, 120, 150, 180, 210, 240, 300 and 360 min for CIP, and 10 sec, 1h, 2h, 3h, 4h, 5h and 6h for TC), the tubes were centrifuged at 4500 rpm for 5 min, the supernatant were filtered (0.2  $\mu\text{m}$ ) and analyzed by UPLC to calculate the un-transformed amount of CIP/TC, and by LC/MS to identify the CIP/TC byproducts.

### 2.4. UPLC and LCMS analysis

CIP aqueous concentrations were determined using an ultra-performance liquid chromatography (UPLC Hclass Waters) equipped with a UV detector and a reversed-phase C18 column (250 mm $\times$ 4.6 mm i.d., 5  $\mu\text{m}$ ). The injection volume of UPLC Hclass was set to 50  $\mu\text{L}$  for each analysis. Two different mobile phases were applied to UPLC for CIP and TC determination. Mobile phase A was an aqueous solution containing 90% ultra-pure water, 10% acetonitrile and 0.1% formic acid. Mobile phase B was 0.1% formic acid in acetonitrile. The volume fraction of solvent B was 10% for the analysis. The mobile phase flow rate was set to be 0.4 mL/min. The detection limit of CIP was 0.03 $\mu\text{M}$  via this method.

The byproducts were, in turn, analyzed with a Waters Ultra LCMS (Acquity UPLC) system using a Waters BEH C18 column (100 mm  $\times$  2.1 mm, 1.7  $\mu\text{m}$ ) and a mass spectrometer. The mobile phase consisted of acetonitrile containing 0.1% of formic acid (solution A) and a mixture of acetonitrile/water 10%/90% containing 0.1% of formic acid (solution B) with gradient 0 min/0% A – 1 min/0% A – 9 min/100% A – 12 min/ 0% A, and a flow rate of 400  $\mu\text{L min}^{-1}$ . An electrospray interface (ESI) was used for the MS measurements in positive ionization mode and full scan acquisition.

### 2.5. Antimicrobial activity assessment of CIP/TC byproducts

An antimicrobial activity assessment of the birnessite generated CIP byproducts was performed by co-incubation in 96-well plates with pure cultured *Pseudomonas veronii*. The byproducts were generated in the presence of birnessite as described above. A total of 7

samples (sample time 0, 1, 2, 3, 4, 5 and 6 h) of byproduct solutions were tested, together with an antibiotic free control allowing at the same time the comparison with the exposure and the recovery of the growth curve. The strain was grown on LB medium overnight at 28°C. An inoculum (100 µL) with a final OD at 600 nm of 0.1 (Spectro, SAFAS) was exposed in triplicate to each byproduct solutions (20 µL stock solution and 80 µL ultra-pure water) except the control where the antibiotic was replaced by water. Negative control was consisted by 100 µL LB and 100 µL pure water. The plates were incubated 24h at 25 °C in a microplate reader (SAFAS Xenius XM, Monaco), continuously stirred (4Hz), measuring the OD at 600 nm every 10 minutes. The same experiment was also done using TC byproducts.

In order to evaluate the antimicrobial activity of different CIP oxidation byproducts, a test was set on pure cultured *Pseudomonas veronii*. CIP oxidation byproducts in this test were obtained via the reaction of 100 mg L<sup>-1</sup> birnessite with 10 µM CIP, and were tested to have 2.5 µM CIP with CIP-Ox1234 after 20 min reaction, and 0 µM CIP with CIP-Ox234 after 5 h. The test was proceeded in 96-well plates. An inoculum (100 µL) with a final OD at 600 nm of 0.1 was exposed in triplicate to each byproduct solutions (20 µL byproduct solution and 80 µL ultra-pure water) except the positive control (the byproduct solution was replaced by 2.5 µM CIP) and negative control (the byproduct solution was replaced by water). The final CIP concentrations were 0 µM (control group and Ox234 group), and 0.25 µM (CIP 0.25 µM group and CIP with byproducts Ox1234 group) separately. The plates were incubated 24h at 25 °C in the microplate reader SAFAS, continuously stirred (4Hz), measuring the OD600 every 10 minutes.

## **2.6. Toxicity assessment of CIP/TC byproducts on denitrification activity**

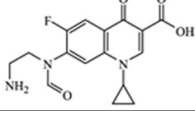
Denitrification batch experiments in closed bottles were carried out in triplicate in 120 mL serum bottles sealed with rubber stoppers (Chen et al. 2021). The main steps of the experiment were described as follows: firstly, the classic mineral medium (designed for denitrifiers) was prepared at pH 7.00 ± 0.05 (composition and concentration see Table S1); the medium was then autoclaved at 121 °C for 20 min and was purged with nitrogen gas for 30 min to remove oxygen; then 10 mL bacterial culture was anaerobically inoculated into 90 mL mineral medium at a final OD600 of 0.1. CIP/CIP-OXs was added into the mineral medium and the final CIP concentration was set at 0.25 µM. Finally, the serum bottles were cultured in an incubator with 180 rpm rotary at 28 ± 0.5 °C in dark. Samples were collected from each serum bottle by sterilized syringe 2 times every day for a total time of 4 days. The same experiment was also done using TC byproducts.

### 3. Results and discussion

#### 3.1. Abiotic transformation of CIP in birnessite suspension

Birnessite-mediated oxidation of CIP (100  $\mu$ M) was monitored in aqueous solution at pH 5. As shown in figure S2, several peaks could be found, suggesting that the oxidation byproducts could be either more hydrophilic or more hydrophobic than CIP. These identified byproducts were grouped due to the retention time and were named CIP-Ox1, CIP-Ox2, CIP-Ox3 and CIP-Ox4. The structure of the byproducts could be deduced according to the m/z value determined via LC-MS (table 1 and figure S3), and their peak intensity changes against reaction time were plotted as figure 2A.

Table 1. LC/MS data for birnessite induced degradation of CIP

Compound name	Retention time (min)	m/z value	Proposed structure
CIP	3.8	334	
CIP-Ox1	3.0	332	
CIP-Ox2	4.7	362	
CIP-Ox3	5.4	334	
CIP-Ox4	5.8	263	

The possible degradation pathway in our reaction system could then be as depicted in figure 1, which is in agreement with previous works (Deng et al. 2017, Xiao et al. 2013, Zhang &Huang 2005, Zhao et al. 2021). The opening of piperazine ring was expected due to the angle stress caused by inductive effect of electronegative fluorine atom. A “double iminium ion” species is likely to act as a reactive intermediate in the present system (Zhang &Huang 2005). Oxidation of this species led to formation of a dialdehyde compound CIP-Ox3 (m/z 362), and two monoaldehyde compounds CIP-Ox1 and CIP-Ox2 (m/z 334). The stability of the three products was different in the presence of birnessite (Fig. 2A). The concentration of CIP-Ox1 gradually

decreased before the concentration of CIP-Ox2 and CIP-Ox3 reached a plateau. All the three products showed a decay trend over reaction time. During degradation of CIP-Ox123, CIP-Ox4 (m/z 263) has accumulated at the same time. Thus, transformation of CIP-Ox123 is supposed to be the source for CIP-Ox4. The abundance of CIP-Ox4 increased over the reaction, suggesting this fully dealkylated product is the final product.

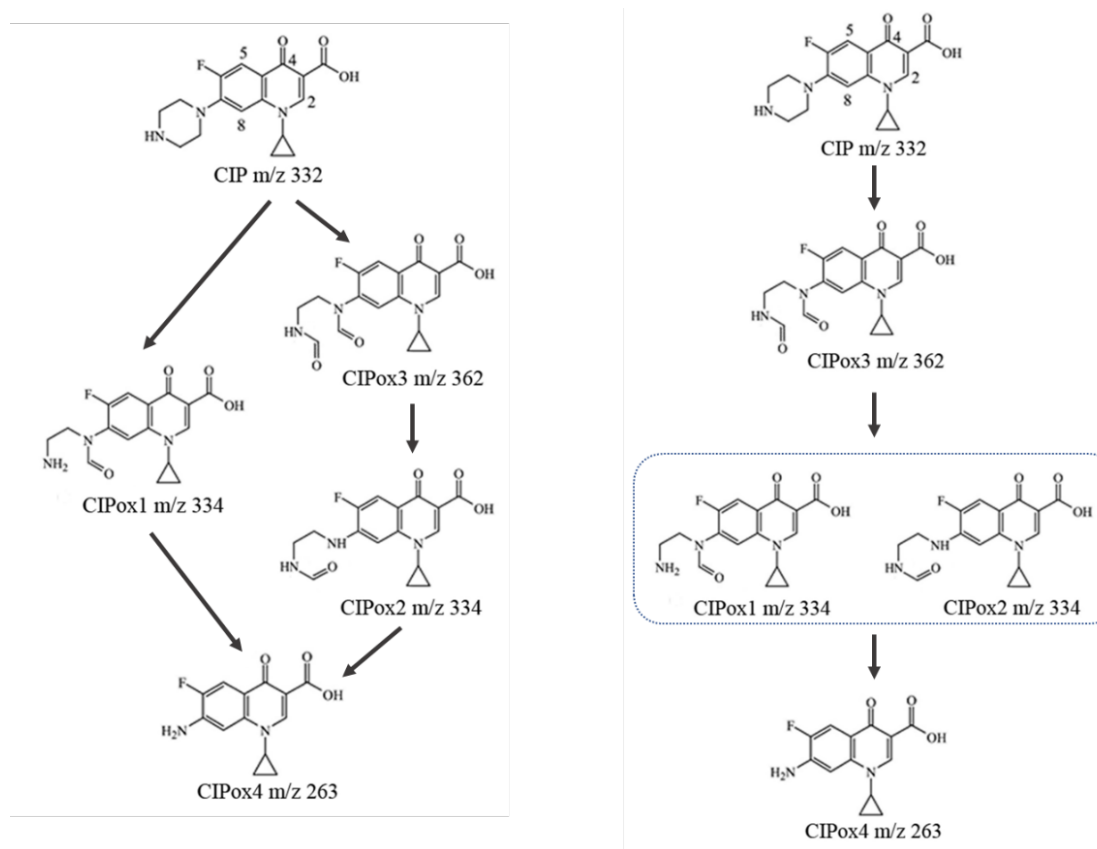


Figure 1. Two proposed ciprofloxacin oxidation pathways in the present birnessite contained system

The fluoro group on fluoroquinolone molecules facilitates cell penetration and gyrase affinity (Peterson 2001, Zhang et al. 2018). Study on oxidation of fluoroquinolones also proposed that the quinolone moiety is the key of fluoroquinolones antimicrobial activity (Dodd et al. 2006). The quinolone core structure with its essential functional groups for gyrase binding remained unaffected in the present birnessite system. The possibility of residual antibacterial activity cannot be ruled out. Moreover, the increased hydrophilicity of CIP-Ox1 may cause higher antimicrobial activity than CIP.

### 3.2. Effect of the mineral induced byproducts on growth of *P. veronii*

The antimicrobial activity of the mineral induced CIP oxidation products was tested on the

growth of the denitrifier *P. veronii*. Figure 2A and 2B shows the change in proportions of CIP and the byproducts over time, showing a decrease of CIP from 93  $\mu\text{M}$  to 5  $\mu\text{M}$  within 6 hours. Within 1 hour, 50% of CIP was transformed/oxidized. The cell viability of *P. veronii* in the presence of the different byproducts, collected at different reaction times, was tested (Figure 2C). Whereas CIP inhibited cell growth, the CIP byproducts seem to alleviate this inhibition, as shown by an increase in growth. As shown in figure 2, samples (0, 1, 2, 3, 4, 5 and 6 h) with different proportion of byproducts have different antimicrobial activities. With the reaction time increases, the concentration of CIP gradually decreased, and the antimicrobial activity also decreased, suggesting that mother CIP has the highest antimicrobial activity.

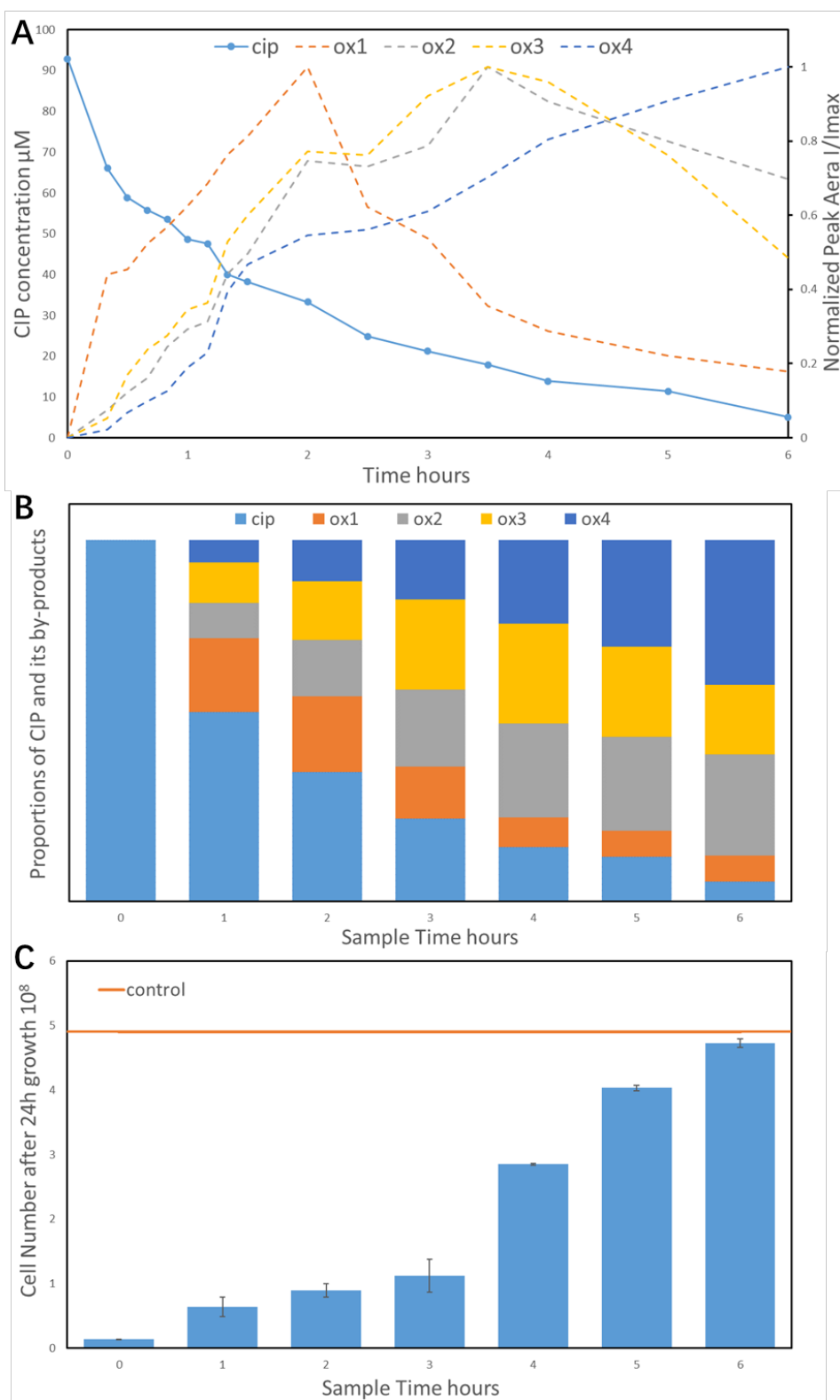


Figure 2. CIP and CIP byproducts against time (A), the proportions of different byproducts from different reaction time (B) and the cell viability test for samples from different reaction time (C), (n=3). The peak area (intensity, I, in figure A) was used to describe the relative concentration changes of CIP byproducts against time, and was normalized by its maximum value during the reaction time (6 hours).

The effects of the different byproducts were then quantitatively evaluated by comparing the cell number within a same culture period. Growth of *P. veronii* in LB medium in the presence of CIP and byproducts are shown in figure 3. Under ideal conditions, in the absence of CIP or CIP byproducts, growth of *P. veronii* follows a classic growth curve with a doubling time of around 5.5 hours, reaching  $4.6 \times 10^8$  cells  $\text{mL}^{-1}$  after 24h. At a concentration of  $0.25 \mu\text{M}$  CIP, cell numbers after 24h ( $2.5 \times 10^8$  cells  $\text{mL}^{-1}$ ) are 46 % lower than in the control. *P. veronii* cultured in the presence  $0.25 \mu\text{M}$  CIP and CIP-Ox1234 reaches cell numbers after 24h of  $1.9 \times 10^8$  cells  $\text{mL}^{-1}$ . These numbers are significantly lower than the control group by 58%, and lower than the CIP  $0.25 \mu\text{M}$  group by 22%. It appears that a mix of CIP and Ox1234 byproducts results in lower cell numbers compared to CIP only, which indicates that the Ox1234 byproducts have additional antimicrobial activity. After a 6-hour reaction with birnessite, the remaining degradation products CIP-Ox2, CIP-Ox3, and CIP-Ox4 did not exhibit any antimicrobial activity and reached cell numbers of  $4.4 \times 10^8$  cells  $\text{mL}^{-1}$  after 24 hours, which were similar to the non-amended control. These results indicate that CIP-Ox2, CIP-Ox3, and CIP-Ox4 are non-toxic and lack antimicrobial activity. In contrast, CIP-Ox1 still retained its antibacterial ability, and inhibited cell growth.

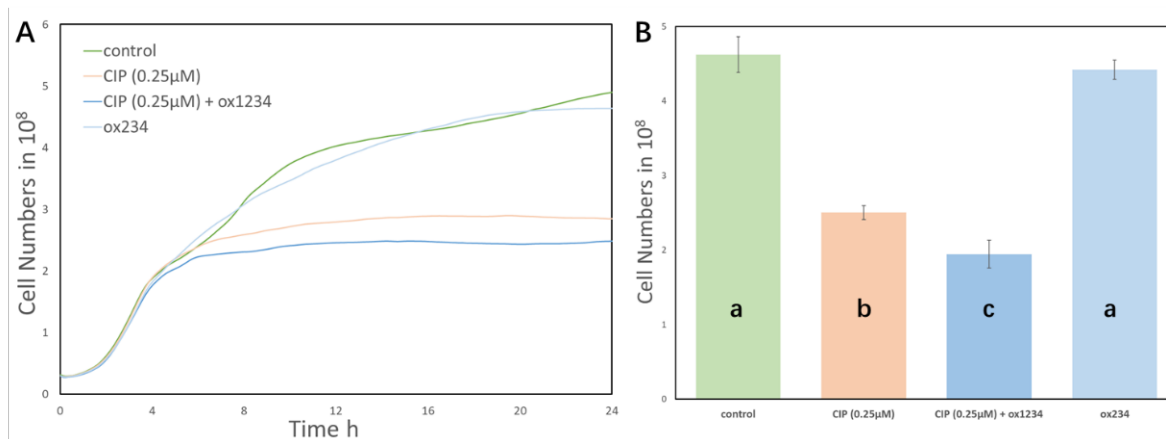


Figure 3. Growth curves (A) and number of cells after 24h growth (B) of *P. veronii* in the absence of CIP (control), in the presence of  $0.25 \mu\text{M}$  CIP,  $0.25 \mu\text{M}$  CIP and byproducts Ox1234 and in the presence of byproducts Ox234. All experiments were done in triplicates. Average cell numbers at different antibiotics concentrations ( $\mu\text{M}$ ) sharing the same letter do not differ significantly at the  $p < 0.05$  level according to t test.

The antimicrobial activity remained on CIP-Ox1 could be partly due to the lack of defluorination in the reaction system, for the reason that the fluoro group on fluoroquinolone molecules facilitates cell penetration and gyrase affinity (Peterson 2001, Zhang et al. 2018). However, it is noteworthy that CIP-Ox2, CIP-Ox3, and CIP-Ox4 also contain the -F group but do not exhibit inhibition of bacterial growth. Among these degradation products, CIP-Ox1 has

higher hydrophilicity, which could be a result of oxidative cleavage of the piperazine ring. It is plausible that the combination of increased hydrophilicity and incomplete oxidation of the piperazine ring contributes to the observed antimicrobial activity of CIP-Ox1, with respect to CIP-Ox2, CIP-Ox3, or CIP-Ox4.

### **3.3. Effect of CIP and byproducts on nitrate and nitrite reduction**

In addition to the effect of CIP and its byproducts on growth of *P. veronii* under optimal conditions (rich medium, oxic conditions), the effects were tested on growth and activity under denitrifying conditions. A denitrifying, mineral medium (table S1) and anoxic conditions were applied and the nitrate reducing activity was monitored. The same solutions containing CIP and/or CIP byproducts were used as for the growth in LB broth medium and oxic conditions.

As shown in figure 4A, cell numbers of *P. veronii* in the control and Ox234 group increased with time and reached  $0.9 \times 10^8$  cells ml<sup>-1</sup> after 87 hours incubation. Meanwhile, the growth of *P. veronii* in CIP group and CIP+Ox1234 group decreased over time. This is mainly because of the antimicrobial activity of CIP. An 33% higher of cells could be observed in CIP+Ox1234 group than in CIP group. Note that the final cell numbers under anoxic denitrifying conditions were 5 to 10 times lower than that in oxic LB medium, due to the lower efficiency of ATP synthesis coupled to the reduction of nitrate to nitrite than coupled to the reduction of oxygen, when NADH act as the electron donor (John &Whatley 1970, Koike &Hattori 1975).

The denitrifying activity, i.e. the change in concentrations of nitrate and nitrite, are shown in figure 4B and 4C. Nitrate concentrations decrease rapidly for all the treatments and consequent production of nitrite is observed at the start of the experiments. Over 85% of the nitrate is reduced into nitrite within 15 hours. No significant difference on the nitrate reduction activity could be observed among different treatments. Nitrate reduction rates match the nitrite production processes. The same trend was observed for cell growth upon this initial stage. After 60 hours incubation, the nitrate amount in all bottles reached a low level: in the control and Ox234 group no nitrate is present, in the others there is still some residual amount (300-400 μM). In contrast to nitrate reduction rates, the intermediate nitrite reduction rates show contrasting behavior (see figure 4C): nitrite reduction process persisted in CIP-free groups (control group and Ox234 group), but ceased in the CIP amended cultures (CIP group and CIP+Ox1234 group), underscoring the key role of CIP in nitrite reduction inhibition. In addition, higher NO<sub>2</sub> amount is detected in Ox234 group than in control group from 40h to 60h. This suggests that byproducts Ox234 might partly retain nitrite reduction inhibitory

capacity.

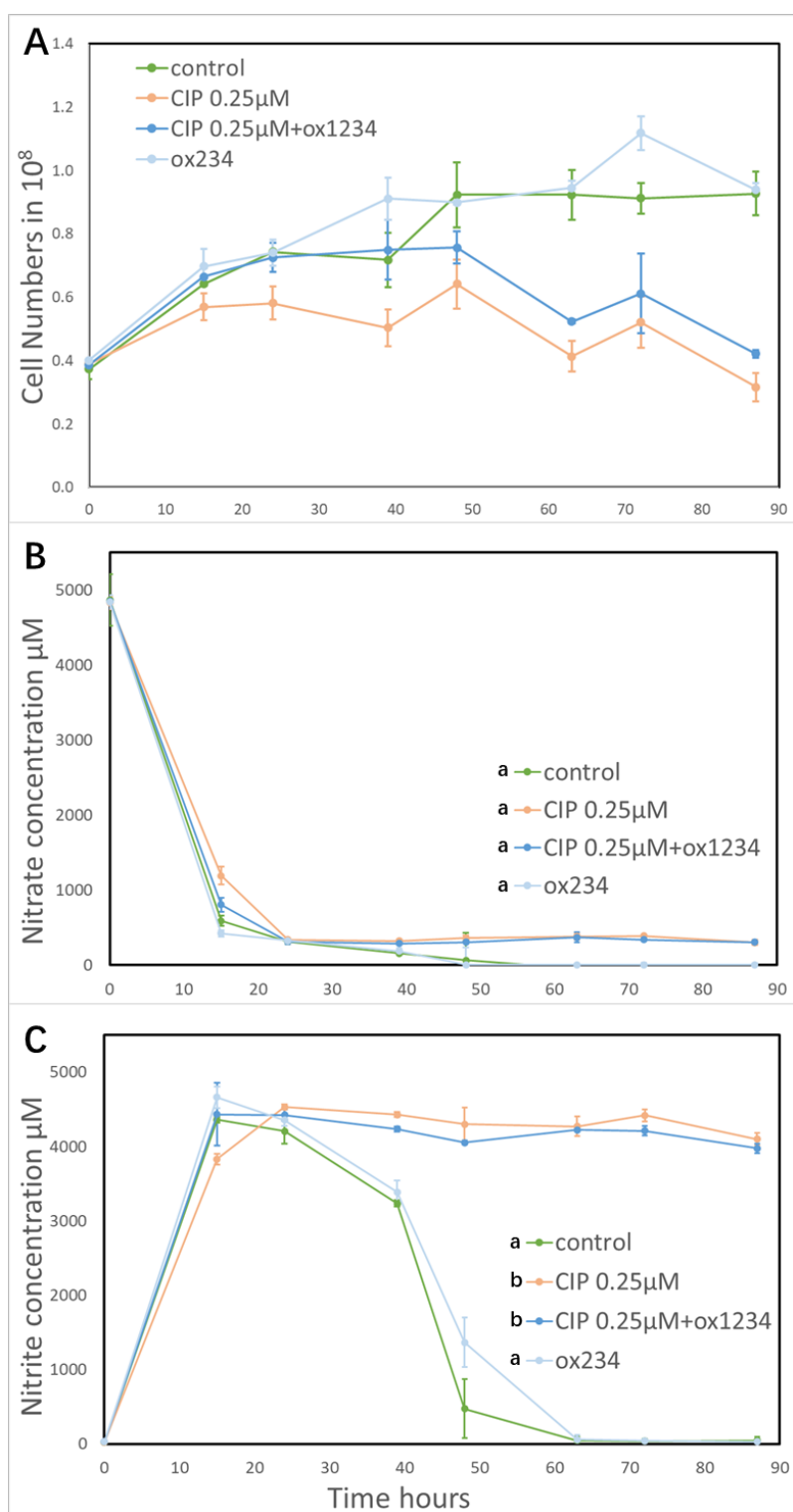


Figure 4. Cell numbers (A) and concentration of nitrate (B) and nitrite (C) of closed bottle tests in the absence of CIP (control), in the presence of 0.25 µM CIP, 0.25µM CIP and byproducts Ox1234 and in the presence of byproducts Ox234. All experiments were done in triplicates. Data sharing the same letter do not differ significantly at the  $p < 0.05$  level according to t test.

In order to take the cell numbers into account, the rates ( $\mu\text{M h}^{-1}$  per cell) of nitrate reduction and nitrite reduction were calculated, and were shown in table 2. Nitrate reduction rates ranged from 405 to 444  $\mu\text{M h}^{-1}$  per cell over the first 15 hours of the experiments. No significant difference between the treatments in nitrate reduction rates was observed over the first 15h (t-test at  $p < 0.05$  level), indicating that neither the presence of CIP nor the CIP byproducts inhibited nitrate reduction during this phase. The nitrate reduction rates were in accordance with the nitrite production rates over the same period (see table 2). The subsequent nitrite reduction rates show significant difference between the different treatments. In the presence of CIP and CIP+Ox1234, the rates ranged between 15.2 and 20.4  $\mu\text{M h}^{-1}$  per cell, significantly lower than the rates in the control (168.5  $\mu\text{M h}^{-1}$  per cell) and in the presence of Ox234 (138.4  $\mu\text{M h}^{-1}$  per cell). However, the control and the Ox234 were not significantly different.

Table 2. The rates of nitrate reduction and nitrite reduction in closed bottles

Time period	0-15 hours		24-48 hours
	Nitrate reduction ( $10^{-8} \mu\text{M h}^{-1} \text{ cell}^{-1}$ )	Nitrite production ( $10^{-8} \mu\text{M h}^{-1} \text{ cell}^{-1}$ )	Nitrite reduction ( $10^{-8} \mu\text{M h}^{-1} \text{ cell}^{-1}$ )
<b>control</b>	444.5 $\pm$ 28.6 <sup>a</sup>	449.7 $\pm$ 3.6 <sup>b</sup>	168.5 $\pm$ 10.3 <sup>c</sup>
<b>CIP 0.25<math>\mu\text{M}</math></b>	425.7 $\pm$ 3.7 <sup>a</sup>	444.9 $\pm$ 8.4 <sup>b</sup>	15.2 $\pm$ 12.7 <sup>d</sup>
<b>CIP 0.25<math>\mu\text{M}</math>+Ox1234</b>	405.9 $\pm$ 7.5 <sup>a</sup>	441.5 $\pm$ 42.3 <sup>b</sup>	20.4 $\pm$ 3.0 <sup>d</sup>
<b>Ox234</b>	422.1 $\pm$ 4.5 <sup>a</sup>	442.5 $\pm$ 13.8 <sup>b</sup>	138.4 $\pm$ 12.1 <sup>c</sup>

\*Data sharing the same letter do not differ significantly at the  $p < 0.05$  level according to t test.

The reduction of nitrate via nitrite to nitrogen gas is carried out by denitrifying enzymes. The nitrate reductase (NAR) in *Pseudomonas veronii* is responsible for the first step, reducing nitrate to nitrite, while the nitrite reductase (NIR) is responsible for the second step (i.e. reducing nitrite to NO and N<sub>2</sub>O). In this study, nitrite reduction rate in CIP amended groups were lower than CIP-free groups, while nitrate reduction rate was kept at a same level. Ox234 group also showed the same trend, though the influences were lower than that of CIP.

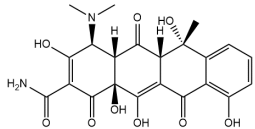
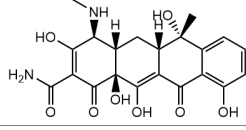
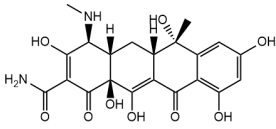
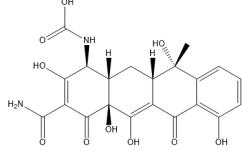
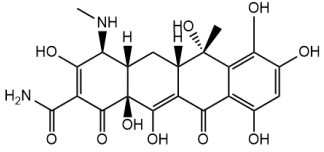
The NARs appear to be little affected by the presence of CIP. It is likely that these enzymes are present and active in the cells at the start of the experiment, allowing the complete reduction of nitrate to nitrite. The further reduction of nitrite into NO, N<sub>2</sub>O and N<sub>2</sub> is inhibited in the presence of CIP, whereas CIP also affects the growth rate of *P. veronii*. Taking into account the cell numbers, nitrite reduction rates are still low, and most likely the antimicrobial effect of CIP on the growth of *P. veronii* impacts the expression of NIRs. NIRs also have shown to be more sensitive facing antibiotics like CIP. In denitrifiers, NAR are stably embedded in the membrane,

whereas NIR are freely distributed in periplasm (Zumft 1997). Similar results were reported in a recent study of Li et al (2021) on denitrification using wastewater and domesticated sludge: lower expression of *nirS* gene (coding NIR) than *narG* gene (coding NAR) was detected under 24 hours exposure of different antibiotics including CIP (Li et al. 2021). Due to their structures, CIP byproducts were inferred to have minor negative impact on nitrate and nitrite reduction. The mechanism of CIP byproducts on NARs and NIRs need to be further studied.

### 3.4. TC oxidation by birnessite

A possible reaction pathway of birnessite induced TC oxidation was proposed based on the identification results of byproducts via LC/MS (Figure S4). TC has higher degradation rate than CIP, thus a first sample point was set as 10 seconds of reaction time. The results showed that almost 60% of total TC was degraded by birnessite during the first 10 second reaction, together with the appearance of 5 different byproducts as identified by LC/MS (see Table 3).

Table 3. LC/MS data for birnessite induced degradation of TC

Compound name	Retention time (min)	m/z value	Proposed structure
TC-Ox1	2.3	459	
TC-Ox2	4.2	431	
TC-Ox3	2.5/4.0	447	
TC-Ox4	5.5	461	
TC-Ox5	1.1/1.5/2.5/4.0	463	

Hydroxylation and demethylation are suggested as the main pathways in the birnessite induced oxidation (Chen &Huang 2011). As shown in Figure S4, the D ring of TC is a phenol moiety which can readily form a phenoxy radical through one-electron transfer with MnO<sub>2</sub>.

The phenoxy radical can be further oxidized by  $\text{Mn}^{\text{IV}}$  to yield a hydroquinone product. The addition of  $-\text{OH}$  group can occur at either the ortho or para position relative to the present phenolic  $-\text{OH}$  group. Demethylation process could be due to the oxidation of the dimethylamino group by  $\text{Mn}^{\text{IV}}$ . The oxidation process first produces a methylene radical. This methylene radical can be further oxidized to an iminium cation, which undergoes rapid hydrolysis to yield N-demethyl derivative and formaldehyde.

Given the chemical structures of byproducts, the antibacterial activity could be then hypothesized. An active tetracycline must possess a linearly arranged DCBA naphthacene ring system with an A-ring diketo substructure and an exocyclic carbonyl or amide group (Fuoco 2012). The A-ring amino group is pivotal for the antibacterial activities. A-ring dimethylamino group with its natural 4S isomer is required for optimal antibacterial activity, while changes to this group decreases antimicrobial activity against Gram-negative bacteria (Doerschuk et al. 1955). According to the LC/MS results, the A-ring dimethylamino group were changed by demethylation process in most of the identified byproducts, suggesting a declined activity of these byproducts compared to the TC mother compound.

### **3.5. Effect of TC and byproducts addition on growth and activity**

The antimicrobial activity of the mineral induced TC oxidation products was also tested on *P. veronii*. Growth of *P. veronii* in LB medium in the presence of TC and byproducts as shown in Figure S5A. Under ideal conditions, the growth curve show no difference between control and TC reacted more than 5 hours, suggesting that antimicrobial activity of these degradation products have been totally removed after 5h reaction with birnessite. As shown in Figure S5B, samples (0, 1, 2, 3, 4, 5 and 6 h, could be seen as with different proportion of TC byproducts) have different antimicrobial activities. With the reaction time increases, the concentration of mother TC decreased sharply, and the antimicrobial activity completely disappeared after 2 hours of reaction time.

In addition to the effect of TC and its byproducts on growth of *P. veronii* under optimal conditions (rich medium, oxic conditions), the effects were tested on activity under denitrifying conditions. The same denitrifying, mineral medium (table S1) and anoxic conditions were applied and the nitrate reducing activity was monitored. As shown in Figure S6A, nitrate concentration decreases rapidly for all the treatments and consequent nitrite production is observed at the start of the experiment (Figure S6B). However, inhibition of TC and its byproducts on nitrate and nitrite reduction of denitrifiers decreases with the reaction

time.

Researches have reported that different antibiotic families could have adverse or inhibitory impacts on denitrification, mainly due to their effects on denitrifying enzymes or genes (Chen et al. 2017, Guo et al. 2020, Li et al. 2021, Su et al. 2019, Zhu et al. 2017). However, there is a lack of knowledge about how antibiotic degradation byproducts would impact the denitrification process, especially under the different concentration range and varied structures in antibiotic-heavily enriched areas, such as sediments. Therefore, the observed results highlight the importance of antibiotic monitoring in riverine zones, and antibiotic accumulations may negatively control on the microbe-driven nitrogen cycles.

#### **4. Conclusions**

Our study showed that CIP/TC and its byproducts can have different effects on the growth and nitrate/nitrite reduction activity of the denitrifier *P. veronii*. The presence of CIP can inhibit bacteria growth and nitrite reduction activity. The first byproduct (CIP-Ox1) still retains its antimicrobial activity, yet it exhibits less activity than the mother CIP. Nitrite reduction activity, rather than nitrate reduction process, of the denitrifier *P. veronii* can be inhibited by CIP and a mixture of byproducts Ox1234. In the case of TC and its byproduct, though the growth of *P. veronii* has been reduced, both nitrate and nitrite reduction activities of *P. veronii* were not significantly inhibited. Therefore, the results of the current study suggest that denitrifiers in sediments can be affected not only by antibiotics like CIP or TC, but also by their degradation byproducts, especially regarding their denitrification activity.

The present study highlights the potential impact of these contaminants on the microbial communities involved in important biogeochemical processes, such as denitrification. Further investigations are needed to evaluate the potential long-term effects of antibiotics and their degradation byproducts on microbial community structure and function in sediments, as well as their potential to influence nutrient cycling and greenhouse gas emissions. Moreover, our study suggests that future research should focus on the identification and characterization of novel microorganisms and metabolic pathways involved in the degradation of antibiotics and their byproducts in sedimentary environments, which may provide new avenues for developing bioremediation strategies for contaminated sediments.

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## **Supporting information**

Mineral induced antibiotic transformation byproducts control on the bacteria growth  
and denitrification activity

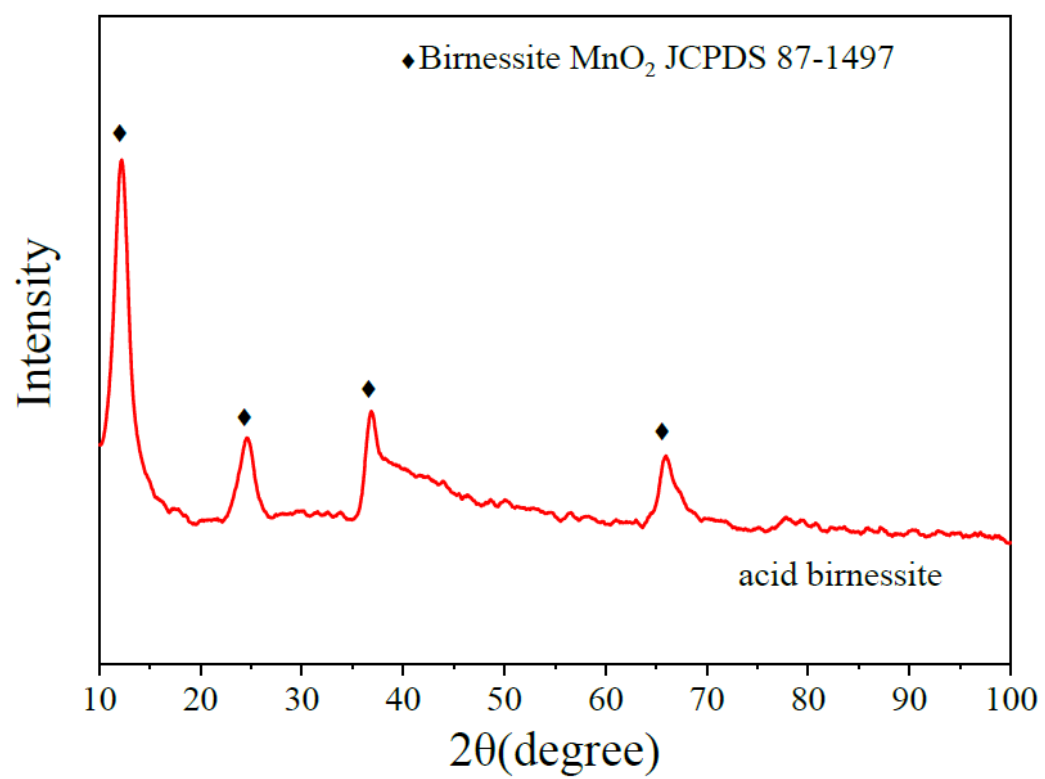


Figure S1. XRD of acid birnessite

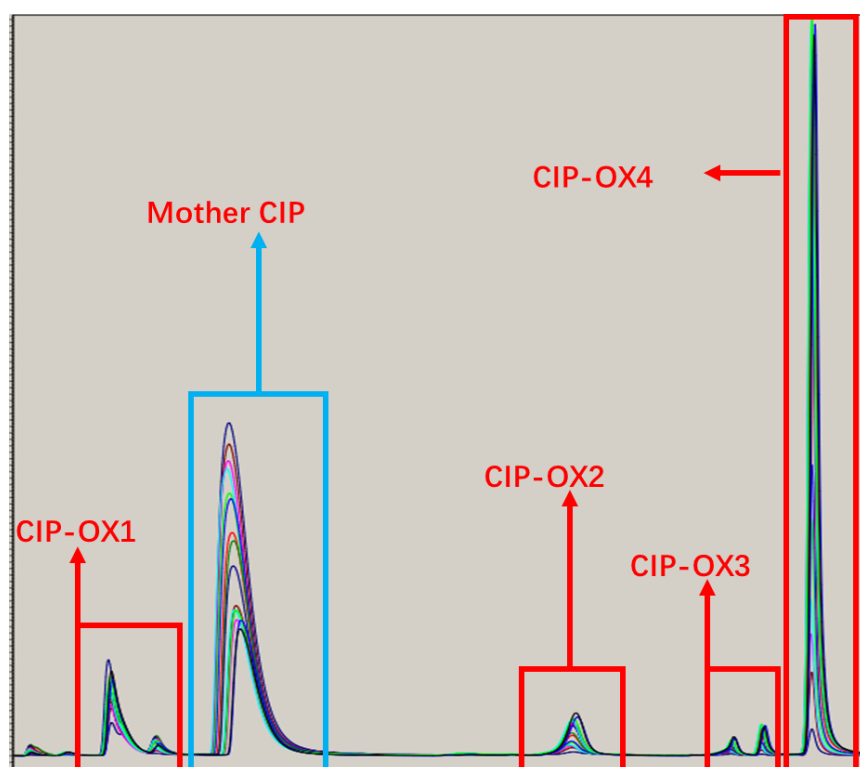


Figure S2. CIP and oxidation byproducts identified by UPLC

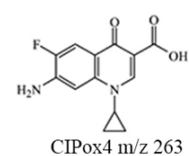
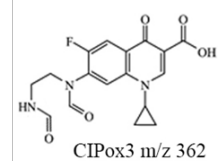
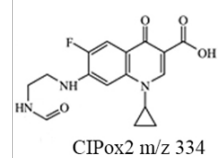
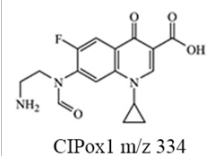
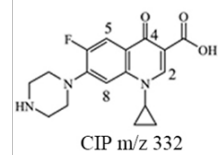
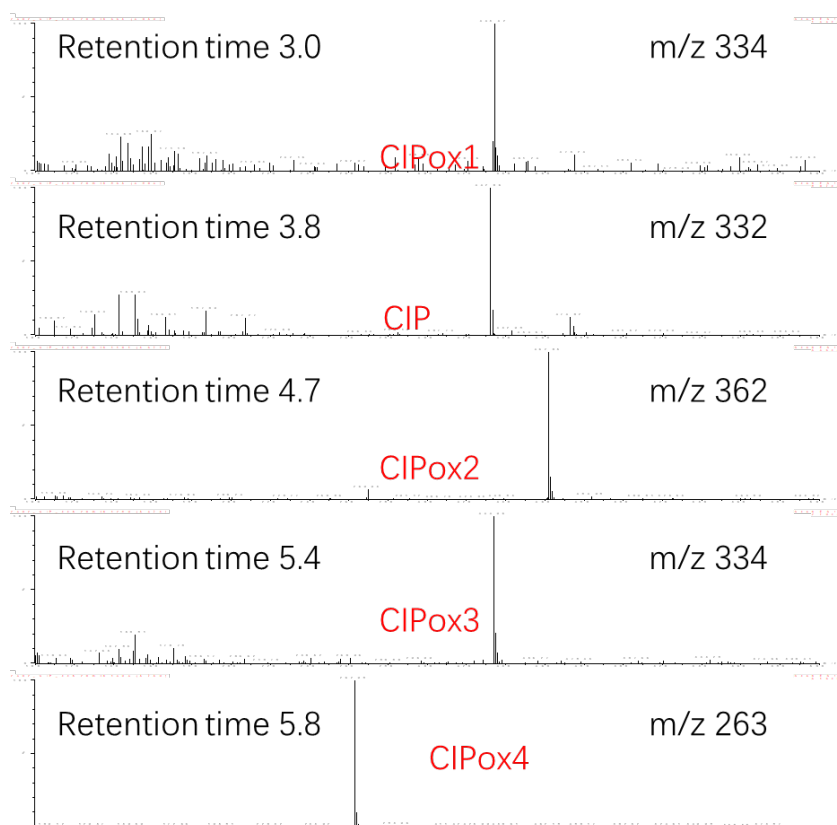


Figure S3. CIP and oxidation byproducts identified by LCMS, and their structures

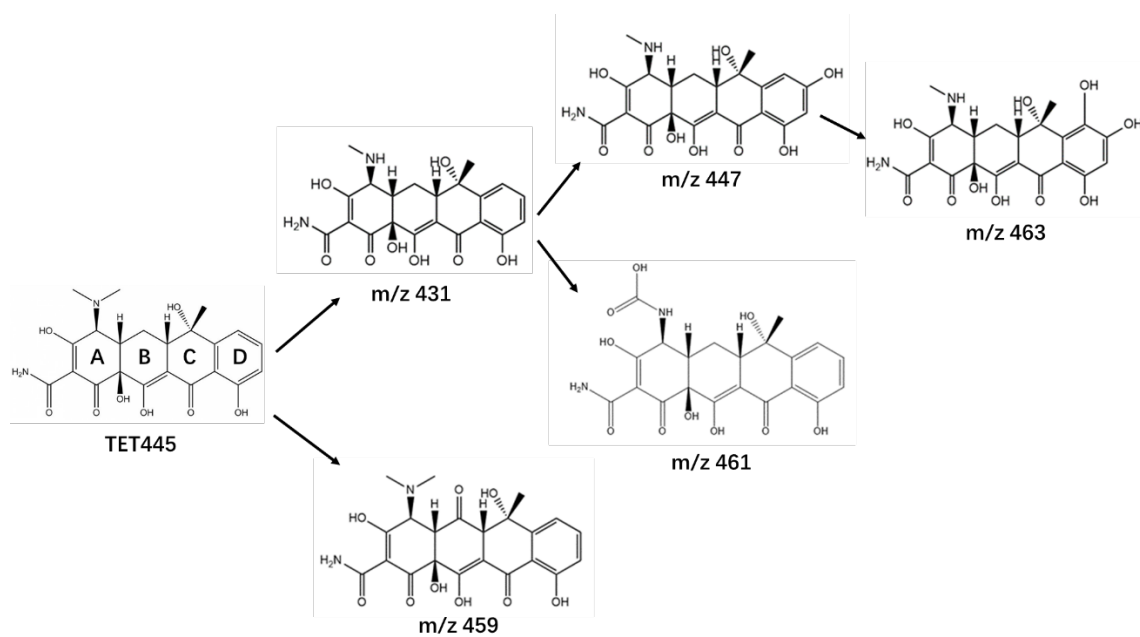


Figure S4. A proposed tetracycline oxidation pathway in the present birnessite contained system

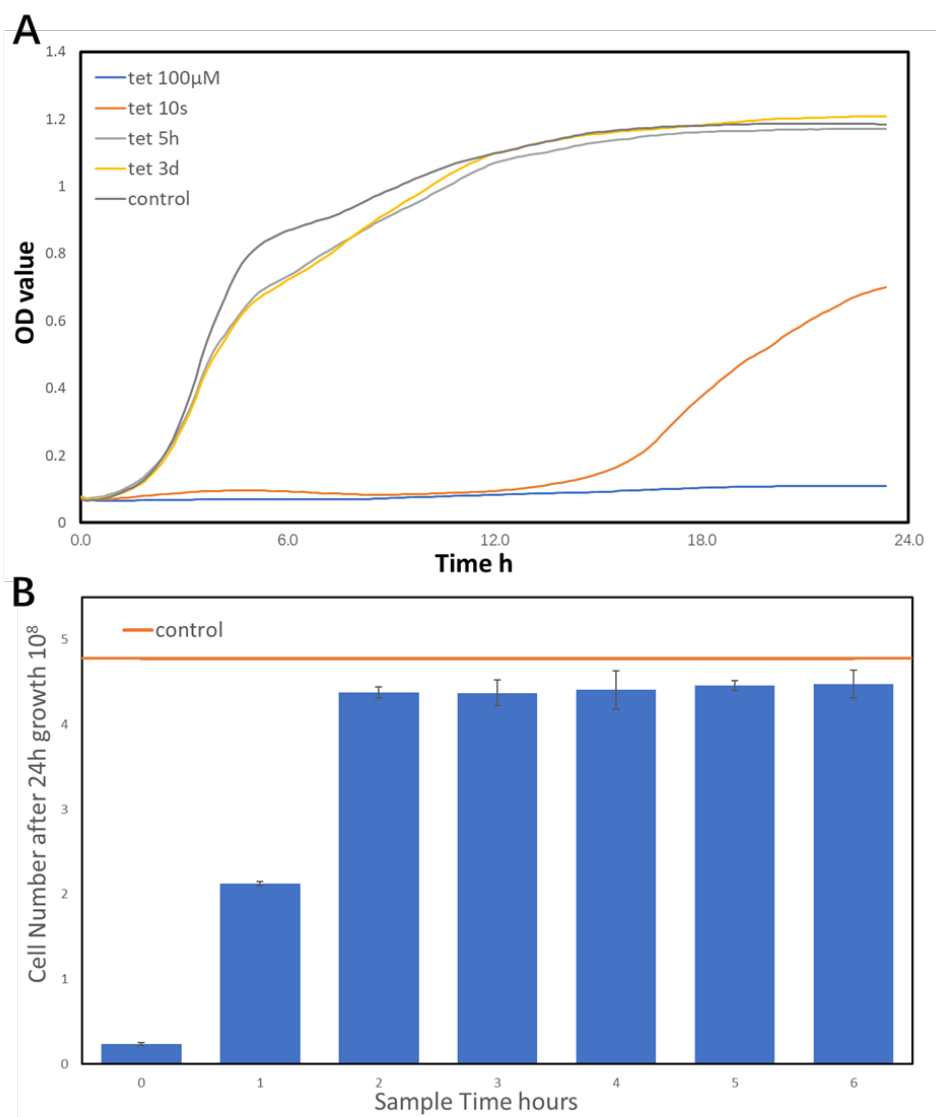


Figure S5. Growth curves (A) and the cell viability test for TC(TET)-birnessite samples from different reaction time (B, n=3).

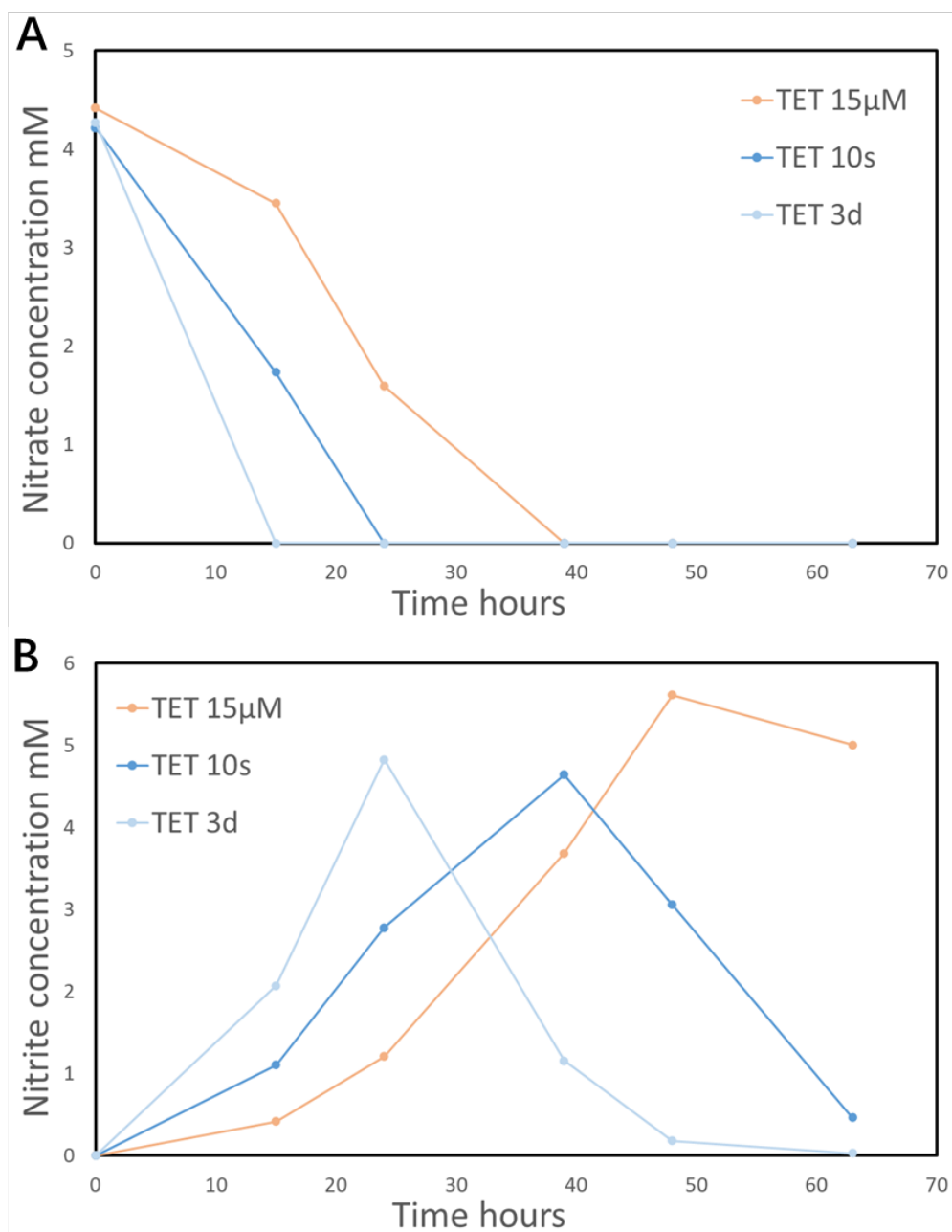


Figure S6. Concentration of nitrate (A) and nitrite (B) of closed bottle tests in the presence of 15  $\mu$ M TC(TET), 10s-reaction of TC-birnessite and 3d-reaction of TC-birnessite samples. All experiments were done in triplicates. Data sharing the same letter do not differ significantly at the  $p < 0.05$  level according to t test.

**Chapter 4**

**Fate and transport of tetracycline and  
ciprofloxacin and impact on nitrate reduction  
activity in coastal sediments from the Seine  
Estuary, France**

**Published (Chen et al 2023)**



## **Abstract**

Fluoroquinolones and tetracyclines are frequently detected antibiotics in aquatic sediments. In this study, the transport of ciprofloxacin (CIP) and tetracycline (TC) was investigated in sediments from the Seine Estuary (France), under nitrate reducing conditions. Dynamic flow experiments showed that although TC and CIP strongly interacted with the sediment components through adsorption and (bio)-chemical transformation, they kept their antimicrobial activities. Less nitrate reduction was observed during the first period of breakthrough, while TC and CIP were absent in the column effluent. Batch experiments with freeze-dried vs fresh sediments showed that adsorption and abiotic degradation are the major removal processes, while microbe-driven transformation is of less importance. Whereas TC is to a large extent chemically transformed and little adsorbed in the sediment, CIP was less transformed and more adsorbed, most likely due to the great reactivity of TC with redox-active mineral surfaces. Our findings show the strong capacity of natural sediment to retain and transform antibiotics, while still maintaining their antimicrobial activity or inhibitory effect of nitrate reducing activity.

**Keywords:** antibiotic; transport; antimicrobial activity; nitrate reduction activity; mobility; sediment.

## 1. Introduction

Fluoroquinolones (FQs) and Tetracycline (TCs) are widely used classes of antibiotics in human and veterinary medicine for treatment and prevention of various infectious diseases (Sarmah et al. 2006). On average, 60 % of the administered doses of tetracycline (Kühne et al. 2000) or quinolones (Vree et al. 1986) are excreted via urine and faeces. These un-metabolized antibiotics can enter the aquatic environment, be transported by water (Kümmerer 2008) and ultimately deposited in soil or sediment (Agunbiade & Moodley 2016, Gibs et al. 2013, Luo et al. 2011, Zhou et al. 2011). Sediments can thus become a major sink for antibiotics, which may increase the risk of the emergence of antibiotic resistant bacteria and the spread of resistance genes.

Monitoring studies of FQs and TCs have been more frequently investigated in the water phase than the sediment one (Harrower et al. 2021). For example, ciprofloxacin (CIP), one of the most used FQs, has been found at levels up to 569  $\mu\text{g Kg}^{-1}$  in sediment (Dinh et al. 2017); tetracycline (TC) concentration reached up to 72.9  $\mu\text{g Kg}^{-1}$  (Yang et al. 2010). For this reason, the fate and mobility of antibiotics in riverine and estuarine sediments are of growing concern. Sorption to sediment constituents, e.g. metal-oxides, clays and organic matter, is a major process affecting the fate and migration of antibiotics in nature (Conde-Cid et al. 2020, Pavlović et al. 2017, Xu et al. 2021). Moreover, redox reactions may occur on environmental surfaces where electron transfer processes may transform the adsorbed compounds. Among these redox active surfaces, Fe and Mn oxides are the most abundant reactive minerals typically present in sediment systems (Martin et al. 2015, Wang et al. 2016, Zhang & Huang 2005). Depending on the molecule properties, half-life of abiotic degradation range from a few days to several months (Boxall 2008).

Furthermore, microbe-driven degradation may play a role in natural attenuation or environmental degradation of antibiotics (Wang et al. 2019). Degradation of tetracycline is one of the TC antibiotic resistance mechanisms. Microorganisms harboring the *tet(X)* gene, encoding tetracycline-degrading monooxygenase are capable of TC degradation (Ghosh et al. 2009). The biodegradability of TC and quinolones, such as ofloxacin and CIP, has been shown to be improved in the presence of carbon sources suggesting co-metabolism (Alexy et al. 2004, Pan et al. 2018). Biodegradation of CIP by consortia or pure bacterial culture has been shown mainly by bacteria originating from waste water treatment plants or conventional activated sludge (Rusch et al. 2019). The biodegradation efficiency of antibiotics by single

microorganisms depends on temperature, pH, carbon source and inoculum; whereas in the environment like soil or sediment, natural adsorption capacity and bacterial community structure could be the main influencing factors (see review of Shao & Wu 2020).

Although different abiotic and biotic processes control the fate of antibiotics in such systems, knowledge about the behavior of antibiotics in more complex environments such as coastal sediments are limited. Most of previous published works have investigated abiotic or microbial degradation of antibiotics separately, and under static conditions (e.g. suspensions), and little is known about their fate and (bio)-chemical transformation under anoxic flow-through conditions. Among the environmental processes naturally occurring in sediment, nitrate reduction is an anaerobic reaction of great importance as it allows a 50% decrease of nitrate pollution of coastal sediments (Galloway et al. 2004). Knowledge on the impact of antibiotics on the nitrate reducing activity of microbes in the coastal sediments is scarce. Therefore, it is important to investigate dynamic flow transport of the most frequently detected antibiotics in nature, coupled to the assessment of their antimicrobial activities within intact real sediment.

In the present work, the fate and mobility of TC and CIP in sediments sampled from north bank of the Seine Estuary, France, was investigated in packed reactors under controlled flow conditions. The sediment sample was first characterized by X-ray Diffraction (XRD), Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES), laser particle size analyzer and elemental C, H, N, S analyzer. The flow through reactors (FTRs) were used to study the mobility of TC and CIP in sediment under nitrate reducing conditions. Inflow solutions of TC and CIP were injected in sediment reactors at a constant flow rate. The inflow solutions contained NaCl and NaHCO<sub>3</sub> to emulate natural coastal environment, and nitrate to determine the nitrate reducing activity. The antimicrobial activity of antibiotics within the sediment was monitored through the determination of nitrate reduction and nitrite production rates using a Gallery™ Plus Discrete Analyzer (Thermo Fisher). Batch experiments with fresh vs freeze-dried sediments were carried out to evaluate respectively biotic and abiotic degradation processes of antibiotics, because freeze-drying is known to minimize bacterial activity (Champagne et al. 1991). Liquid chromatography (LC) combined with UV and/or mass spectrometry (MS) was used to quantify the antibiotics and to identify potential degradation byproducts. The present work will improve the understanding of the behavior of TC and CIP in coastal sediments, and thus has important implications in assessing ecotoxicological risks related to these emerging compounds in the environment.

## **2. Material and Methods**

### **2.1. Sediment sampling and characteristics**

Sediment samples were collected, from north site in Seine Estuary, Saint-Vigor-d'Ymonville, France, in the estuarine section, in September 2020 (Fig. S1). All the sediments collected were composed of fine-grained anoxic mud with a dark gray appearance. The sediment was then stored in aluminum coated containers at a 4°C fridge prior to use. The fresh sediment water had a pH of  $8.04 \pm 0.03$  (average of 10 samples).

Elemental analyzer (Shimadzu TOC V-CSH analyzer) showed that the sediment had an average total organic carbon (TOC) content of 1.49%, and total nitrogen content (TON) of 0.13%. Laser particle size analyzer (Malvern) analysis showed the natural sediment had an average particle size of  $56 \pm 1 \mu\text{m}$ , with a high proportion of  $70 \pm 1 \%$  of 2-63  $\mu\text{m}$  size (classified as silt based on ISO 14688-1), and a minor proportion of  $5 \pm 1 \%$  below 2  $\mu\text{m}$  size (classified as clay based on ISO 14688-1). According to XRD analysis, the sediment was mainly composed of quartz with traces of metal oxides (not shown).

Acid digestion of the sediment followed by ICP-OES measurements showed high concentrations of Ca with respect to Al, Fe and Mg. Cu, Ni, Cd and Co were below the detection of the ICP-OES. A sequential extraction procedure was also conducted according to a previous reported method (Ure et al. 1993). In the first step, the acid-soluble fraction (carbonate and exchangeable fraction) was extracted with HCl 0.1mM. HCl (10 mM, pH 2) was then used in the second step to digest metals linked to the amorphous fraction. Total acid digestion was finally carried out using 50 % aqua regia (3:1 HCl: HNO<sub>3</sub>) to completely remove total metal content. The supernatant was analyzed by ICP-OES. The characteristics of the Seine sediment are shown in Table S1.

### **2.2. Flow-Through Reactors**

The setup of the Flow-Through Reactors (FTR) was designed to investigate the effect of natural sediment on breakthrough behavior of selected antibiotics (TC and CIP) when continuously supplied with a defined input solution at a low flow rate. The FTR system consists of 2 square blocks placed on and under a plexiglass column. Two filters were placed on the inner face of the blocks: a thick glass fiber filter (PALL) to homogenize the inlet of the solution and a PVDF filter (Millipore) with 0.2 $\mu\text{m}$  porosity to prevent bacteria from entering and exiting the reactor. A peristaltic pump (Gilson minipuls3) supplied the solution continuously into the reactor. The

tubes connecting the bottles to the reactors were made of Tygon, to avoid absorbing solvents. A scheme of FTR is shown in Fig. S3 and described in more detail in previous study (Laverman et al. 2006).

Briefly,  $38 \pm 2$  g of fresh natural sediment (well saturated) was packed into plexiglass columns of 4.2 cm internal diameter, 2 cm height, and 27.7 ml internal volume. After packing to a uniform bulk density ( $1.37 \pm 0.07 \text{ g cm}^{-3}$  for fresh sediment), the column was supplied continuously upward with a synthetic input solution at a constant flow rate (2 ml per hour). The basic input solutions were sterile saline water (1% NaCl, 2.4 mM  $\text{NaHCO}_3$ ) supplemented with 5 mM of nitrate ( $\text{KNO}_3$ ) at pH 8, flushed with nitrogen to remove oxygen in the air. The water content of the sediment in FTR was relatively constant with an average value 44 % (average of 20 samples). The pore volume of FTR column ( $\text{PV}=16.8 \text{ mL}$ ) was estimated by weight difference between the fully saturated and freeze-dried sediment. The flow through experiments were duplicated for each experimental set: control group (no CIP or TC), TC ( $4.5 \mu\text{M}$  TC), CIP ( $3.0 \mu\text{M}$  CIP) and MIX ( $5.8 \mu\text{M}$  TC and  $0.8 \mu\text{M}$  CIP). The tested concentrations of TC and CIP were chosen based on the definition of break points between sensitive and resistant ( $9.0 \mu\text{M}$  TC and  $3.0 \mu\text{M}$  CIP) for bacteria, according to the EUCAST and CLSI-potency NEO-SENSITABS™ User's Guide 2013, when applied individually and based on Predictive Environmental Concentration according to DeVries and Zhang (2016) when applied as mixture.

Tracer experiments using bromide ( $\text{Br}^-$ ) showed classical shape, i.e. sigmoidal shape with no big tailing, as reported in a previous work (Pallud et al. 2007). As expected, a small bed length with respect to column diameter suggests that both advection and diffusion may occur in the FTR system. Peclet number ( $\text{Pe}$ ) can be then calculated using the equation  $\text{Pe}=vL/D$ , where  $v$  is the water velocity ( $\text{cm s}^{-1}$ ),  $L$  is the bed length of the FTR column, and  $D$  is the molecular diffusion coefficient of tracer. The water velocity  $v$  was obtained via  $v=q/\theta$ , with  $q$  the Darcy flux ( $\text{cm s}^{-1}$ ) and  $\theta$  the porosity. If we take the molecular diffusion for the bromide ion ( $2.0 \pm 0.2 \cdot 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ ) (Bowman 1984, Shackelford & Daniel 1991, Willingham et al. 2004),  $\text{Pe}$  lies at around 4, thereby underscoring the occurrence of convection and diffusion processes.

### 2.3. Batch Experiments and analyses

Different extraction methods were applied to TC ([1 M NaCl: 1 M oxalic acid: ethanol (25:25:50)]) or CIP ([2% ammonia in methanol + 2% formic acid in methanol + pure methanol]). The detailed operation steps are given in the supporting information. The antibiotic extraction efficiency from sediment was tested to be  $71 \% \pm 4 \%$  for TC, and  $80 \% \pm 3 \%$  for CIP.

Fresh (3.6 g) or freeze dried (2.0 g) sediment and 25 mL TC (4.1  $\mu$ M) or CIP (3.0  $\mu$ M) and nitrate (5 mM  $\text{KNO}_3$ ) containing sterile saline water (171.1 mM NaCl, 2.4 mM  $\text{NaHCO}_3$ ) were mixed in 50 mL tubes. The tubes were flushed with nitrogen, in order to simulate the same condition as FTR. The tubes were then covered by aluminum and incubated in an automatic shaker, at 250 rpm under room temperature. After shaking for a certain time (2 min, 1 week, 2 weeks, and 3 weeks), the tubes were centrifuged at 4500 rpm for 20 min, and the supernatants were analyzed by UPLC to measure and calculate the un-transformed amounts of CIP and TC. Subsequently, the pellet (solid phase) left in the tubes were freeze dried and extracted by the specific extraction method of TC or CIP described in supporting information. All treatment and analysis were done in triplicate.

CIP and TC aqueous concentrations were determined using an ultra-performance liquid chromatography (UPLC Hclass Waters) equipped with a UV detector and a reversed-phase  $\text{C}^{18}$  column (250 mm $\times$ 4.6 mm i.d., 5  $\mu$ m). The injection volume of UPLC Hclass was set to 50  $\mu$ L for each analysis. Two different mobile phases were applied to UPLC for CIP and TC determination. Mobile phase A was an aqueous solution containing 90% ultra-pure water, 10% acetonitrile and 0.1% formic acid. Mobile phase B was 0.1% formic acid in acetonitrile. The volume fraction of solvent B was 10% for the analysis. The mobile phase flow rate was set to be 0.4 mL/min. The detection limit of CIP and TC were respectively 0.03  $\mu$ M and 0.05  $\mu$ M via this method.

## **2.4. Extraction Efficiency Determination**

Two grams of fresh sediment and 25 mL of compound/antibiotic solution (4.5  $\mu$ M TC or CIP) were applied to each 50 mL tube. The tubes, covered by aluminum foil, were shaken at 250 rpm at room temperature with an automatic shaker (IKA Orbital Shaker). After shaking for a certain time (1 min, 20 min, 40 min, 1 h, 2 h, 4 h, 8 h, and 24 h), the tubes were centrifuged at 4,500 rpm for 20 min, and the supernatants were analyzed by an ultra-performance liquid chromatography (UPLC Hclass Waters) to calculate the un-transformed amount of ATBs. For the next step, the supernatants were removed, the solid phases left in the tubes were freeze dried and kept for further extraction.

Different extraction methods were applied to TC ([1 M NaCl: 1 M oxalic acid: ethanol (25:25:50)]) or CIP ([2% ammonia in methanol + 2% formic acid in methanol + pure methanol]). Each group was done in triplicates. ATB concentrations were determined using UPLC Hclass (Waters) as described in section 2.3.

### **Sediment Extraction for TC**

Optimizing sediment extraction efficiency for TC was performed using a specific method (Sassman & Lee 2005). Two grams of fresh sediment were mixed with 4.5  $\mu$ M TC. Following the removal of the supernatant and freeze drying as described in the manuscript, section 2.2.1, TC was extracted from sediment with 25 mL of [1 M NaCl: 1 M oxalic acid: ethanol (25:25:50)] and shaken at 250 rpm for 20 h. The tubes with extraction solution were centrifuged at 4,500 rpm for 20 min. Finally, the supernatants were analyzed by UPLC.

### **Sediment Extraction for CIP**

A 3-step method specific for CIP [2% ammonia in methanol + 2% formic acid in methanol + pure methanol] was set to extract CIP from the sediment. Two g of fresh sediment were incubated with 4.5  $\mu$ M CIP. Following the removal of the supernatant and freeze drying, sediment was extracted with the following steps: 1) add 10 mL 2% ammonia in methanol, mix by ultrasonic for 15 min and 250 rpm shaking for 30 min, centrifuge at 4,500 rpm for 20 min; 2) remove the supernatant, add 10 mL 2% formic acid in methanol, mix by ultrasonic vibration for 15 min and 250 rpm shaking for 30 min, centrifuge at 4,500 rpm for 20 min; 3) remove the supernatant, add 5 mL pure methanol, mix by ultrasonic for 15 min and 250 rpm shaking for 30 min, centrifuge at 4,500 rpm for 20 min. Finally, the 3 supernatants were combined and analyzed by UPLC.

## **3. Results and discussion**

### **3.1. Mobility behavior of TC and CIP in FTR**

Breakthrough experiments of TC and CIP were conducted at pH 8 (buffered by the presence of  $\text{NaHCO}_3$ ) and a low flow rate ( $33 \mu\text{L min}^{-1}$ ) to avoid kinetic limitations under flow-through conditions (Fig. 1). For all systems, the outflow pH changed very slightly, and stayed at  $8 \pm 0.1$ . The solute breaks out at around 25 pore volumes (PV), for both TC and CIP, and regardless of the injection scenario (single or binary). Then, the outflow concentration increases gradually with a long tailing, but does not exceed  $C/C_0 \sim 0.15$ , even after an injection of 75 PV (about 600 hours under the current flow rate). This suggests that the sediment has a great capacity to remove TC or CIP from aqueous phase, through for instance sorption to sediment constituents and/or (bio)-chemical transformation.

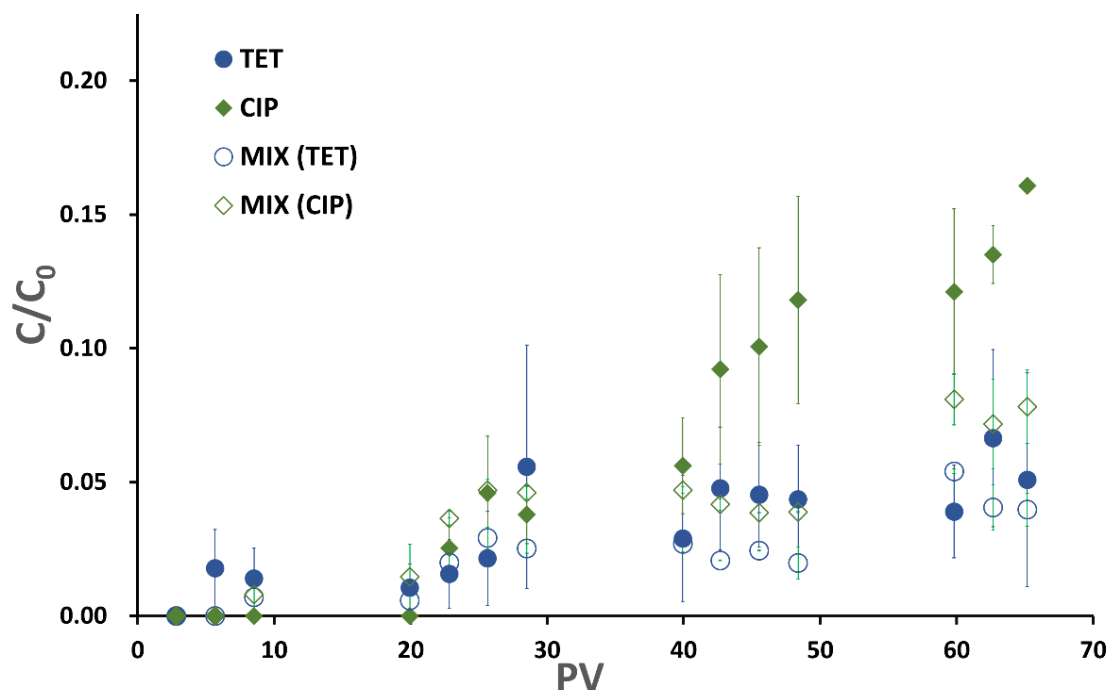


Figure 1. CIP and TC breakthrough curves (BTC) from FTR experiments. Experimental conditions: pH 8 (2.4 mM  $\text{NaHCO}_3$ ), nitrated saline water (1% NaCl, 5mM  $\text{KNO}_3$ ), and flow rate  $33 \mu\text{l min}^{-1}$ . TC and CIP inflow concentrations ( $C_0$ ): TC ( $4.5 \mu\text{M}$ ), CIP ( $3.0 \mu\text{M}$ ), and MIX ( $5.8 \mu\text{M}$  TC and  $0.8 \mu\text{M}$  CIP). The error bars are standard deviations for duplicates. PV, pore volume

To determine the mass balance, an extraction of the sediment after the breakthrough experiment was conducted and compared to the removed amount calculated from the breakthrough curve (BTC) of each compound. Mass balance calculations indicated that  $51 \pm 2\%$  of removed TC and  $32 \pm 3\%$  of removed CIP were not recovered independently on the injection mode (single or mixture). This suggests molecular transformation of TC and CIP within the sediment during the dynamic flow experiments.

Previous reports have shown that microbial abundance and diversity within sediments can be altered by the presence of antibiotics in inflow solution (Fan et al. 2020, Girardi et al. 2011, Liu et al. 2016). To evaluate the impact of TC and CIP on the nitrate reducing activity within the sediment, the BTC of nitrate and nitrite were also determined versus PV (Fig. 2). Alteration of the nitrate reduction and nitrite production activity are mainly observed during the first 12 PVs of the breakthrough experiment. As shown in Fig.2 and Table S2, less nitrate flowed out from the control group than from those with addition of antibiotics. This indicates that more nitrate was consumed in the sediment without antibiotics, i.e. the control group ( $1458 \pm 202 \mu\text{M}$ ) compared to the antibiotic amended sediments (TC  $1135 \pm 182 \mu\text{M}$ , CIP  $1293 \pm 282 \mu\text{M}$ ,

MIX  $1085 \pm 268 \mu\text{M}$ ), implying that nitrate reduction activity in the sediment during this stage was inhibited. This inhibition is approximately 30% for TC and 15% for CIP compared the control. This inhibition was only observed during the first period of the experiments (up to 12 PV).

The intermediate nitrite production also shows differences, nitrite production in the control group ( $299 \pm 190 \mu\text{M}$ ) was 2-3 times higher than that in antibiotic added groups (TC  $117 \pm 101 \mu\text{M}$ , CIP  $113 \pm 102 \mu\text{M}$ , MIX  $98 \pm 58 \mu\text{M}$ ). For the sediments exposed to TC the nitrite production remains higher throughout the experiment than in the other treatments (see Fig. 2). Overall, the lower nitrate reduction and nitrite production rates in the sediments exposed to the antibiotics indicate an inhibition on the microbial activity. Even though TC concentrations are low in the outflow, its presence impacted nitrate reducing and nitrite producing activity. Similar experiments showed a lack of effect due to TC in river sediments, with TC concentrations up to  $22.5 \mu\text{M}$  (Roose-Amsaleg et al. 2013). Other studies, however, showed significant decreases of denitrification rates from 30% at  $1.7 \mu\text{M}$  TC to 70% at  $17 \mu\text{M}$  TC in soil samples (Shan et al. 2018), or 75% at  $22.5 \mu\text{M}$  TC in sediment samples (Zhang et al. 2019). These differences in effect of TC on nitrate reduction could be due to a more adapted microbial community, i.e. presence or absence of resistance, or sediment characteristics impacting the availability of TC.

The effect of CIP (quinolones) on nitrate reduction activity is in line with observations in river sediments, with a partial inhibited nitrate reduction rate by 40% (Yan et al. 2013) by  $200 \mu\text{M}$  flumequine (FLU) from the quinolones family. Elevated CIP concentrations ( $90 \mu\text{M}$ ) have shown to inhibit nitrate reduction rates up to 65% in active sludge (Yuan et al 2021) or 10% at  $6 \mu\text{M}$  CIP in WWTP sludge samples (Yi et al 2017).

In the treatment with both TC and CIP (MIX), no significant difference compared to the single application was observed regarding the nitrate reduction and nitrite production rates. After injection of  $\sim 50$  PV, no inhibition was observed as nitrate reached complete breakthrough and no nitrite was formed (Fig. 2). The reaching of complete breakthrough of nitrate indicates that nitrate reduction activity has ceased. This could be attributed to the leaching of organic carbon (See Fig. S4), as the same behavior was observed in all systems including the control. The DOC in the outflow of the reactor is the result of (sediment) organic carbon degradation due to microbial activity, including nitrate reduction. The decrease in DOC with time is most likely due to the fact that the available organic carbon decreases over time and no additional carbon was added during the experiment. The quick decrease in DOC observed over the first 12 PVs is

consistent with the period in which denitrifiers were more active (as shown in Fig 2). Indeed, a decrease in organic carbon within the sediment may affect the microbial and nitrate reducing functioning as organic carbon is required for nitrate reducing activity (Payne 1973).

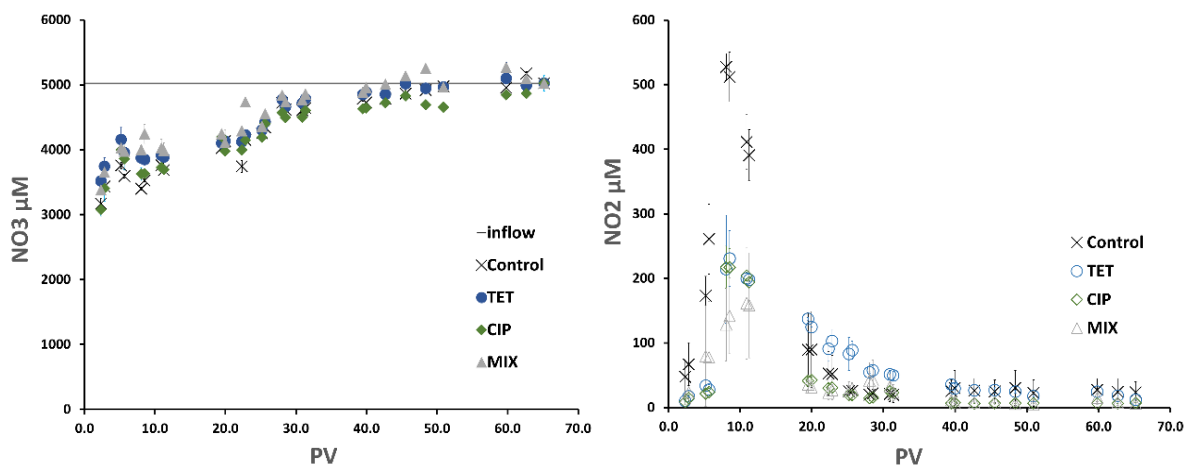


Figure 2. Nitrate and nitrite breakthrough curves. Experimental conditions: pH 8 (2.4 mM NaHCO<sub>3</sub>), nitrated saline water (1% NaCl, 5mM KNO<sub>3</sub>), and flow rate 33 μl min<sup>-1</sup>. TC and CIP inflow concentrations: TC (4.5 μM), CIP (3.0 μM), and MIX (5.8 μM TC and 0.8 μM CIP). The error bars are standard deviations for duplicates.

### 3.2. Transformation of TC and CIP under batch conditions

To check whether the molecular transformation of TC or CIP may take place in the investigated sediment, batch experiments were conducted over 3 weeks in aqueous suspension under similar conditions as for reactor experiments at pH 8. Since freeze-drying is known to inhibit the bacterial activity (Champagne et al. 1991), biotic (microbe-driven process) and abiotic (mineral-induced reactions) degradations can be assessed through comparison of removal amounts in fresh vs freeze-dried sediment.

As observed in the FTR system, strong adsorption of TC and CIP can be achieved for both fresh and freeze-dried sediments (Fig. 3). Extraction was then conducted at different reaction times to determine the mass balance, and thus to calculate the amount of transformed (unrecovered) compound (Fig. 3). This amount increased with reaction time, and is slightly higher for the fresh sediment, suggesting the occurrence of both abiotic and biotic degradation in the sediment.

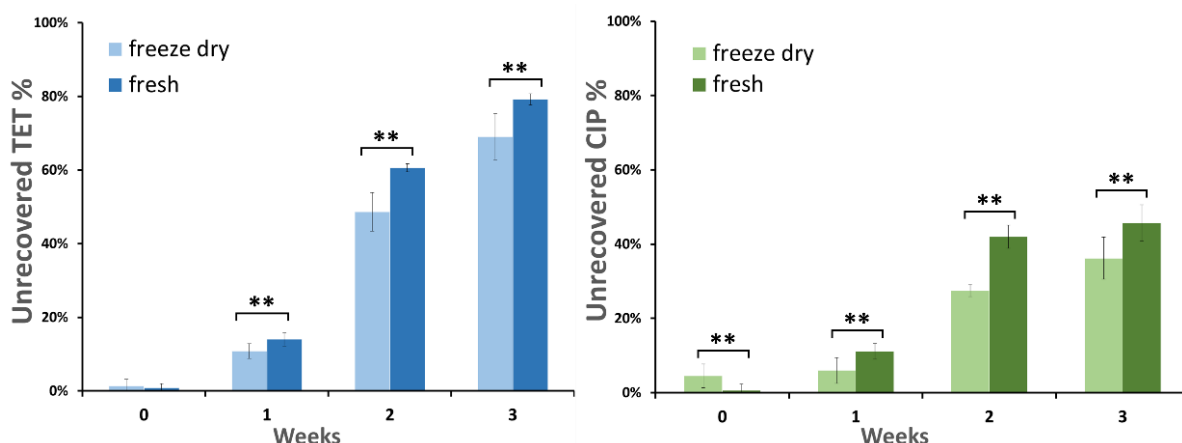


Figure 3. Comparison of unrecovered ratio of TC and CIP to total aqueous removal amount, in the presence of freeze-dried sediment and fresh sediment. P-value statistical significance testing: when set  $\alpha=0.05$ , \*\* represents  $p<0.05$  (a significant difference could be stated between two tested groups). The error bars are standard deviations ( $n=3$ ).

After three weeks of reaction time, around 70 % of TC and 40 % of CIP were transformed. In the fresh sediment, a slight increase in transformation was observed, as compared to the freeze-dried sediments. If we suppose that this difference, though slight, can be attributed to biological-driven degradation, we can determine the contribution of each process in the total removal of compound in batch experiments (Fig. S5). In the present work, aluminum foil was used to prevent photolysis, nitrogen gas flush was applied to inhibit aerobic biodegradation, and consequently anaerobic microbes like denitrifiers could potentially contribute to the biodegradation of TC and CIP. The biological-driven degradation for both TC and CIP was approximately 10%, confirming the minor contribution of this process. Different works using different sediment and soil systems reported different biodegradation rates for CIP or TC, thereby highlighting the key role of sediment characteristics and microbial community structure in determining the antibiotic removal. For instance, higher biological degradation in sediments/soils for TC was previously reported (Chang &Ren 2015, Pan &Chu 2016). For example, Chang et al. (2015) reported 79% decrease of TC in natural sediments, with 6.8% in autoclaved sediments, representing the abiotic transformation. Pan et al (2016) found that after 21 days of anaerobic incubation, 46.8% of total TC loss in non-sterilized soil versus 24.4% in a sterilized group. Comparable to the 10% biotic transformation in the investigated coastal sediments, low biodegradation rates were previously found for CIP. Zhang et al. (2012) showed that 13% of CIP can be biodegraded whereas Thuy et al. (2014) found only 5% of total CIP could be removed in sterile vs non-sterile sediment system.

Overall, the results of the current study suggest that adsorption and abiotic degradation are

the predominant processes for the removal of both TC and CIP, probably due to the characteristics of the investigated sediment.

### **3.3. Difference in TC and CIP behavior within the sediment**

Although the mass balance showed that CIP is more adsorbed and TC is more chemically transformed within the sediment, the affinity of both compounds to the sediment can be considered similar (i.e. sum of adsorption and chemical transformation is close) (see Fig. S5). It is known that the fate of antibiotics is often tied to their affinities to organic matter (Sithole & Guy 1987, Tolls 2001) and mineral particles (Nowara et al. 1997, Zhang et al. 2010) of sediments.  $\pi$ - $\pi$  electron donor-acceptor interactions, electrostatic interactions, H-bonding, and hydrophobic interactions, and surface complexation seem to be the most prominent mechanisms for their binding with sediment minerals and organic matter (Zhu et al. 2004). These interactions are mainly affected by pH, sediments properties, and structural characteristics of the target compounds. Fluoroquinolones such as CIP are electron donor-acceptor compounds (Vasudevan et al. 2009) with the piperazinyl group serving as the electron donor and carboxylic group as the electron acceptor in the neutral and zwitterion forms (Fig. S6a). TC contains phenolic and carboxylic groups as the electron acceptor and the N-group as the electron donor, and has three  $pK_a$  (Fig. S6a). In the present work, the investigated sediment has a stable pH at 8, which could result in certain pH-dependent speciation of TC and CIP. As shown in Fig. S6b, both CIP and TC exist under zwitterion form at pH 8, which could be adsorbed onto charged mineral surfaces (Vasudevan et al. 2009, Zhao et al. 2012). The organic matter can also improve the total removal of compounds through adsorption process. For instance, 95 $\pm$ 5% of oxytetracycline and 30 $\pm$ 10% of tylosin were found removed by a sand-based soil with a TOC at 1.4% (Rabølle & Spliid 2000), whereas high TC adsorption to a marine sediment has been attributed to natural organic matter, though the investigated sediment contained only 1.3-1.9% of TOC (Xu & Li 2010). In the present work, we can suppose that 1.5% of sediment organic carbon may contribute to the total sorption of TC and CIP. Other works investigated the adsorption of antibiotics onto a sediment containing a similar organic content (1% TOC), and found high removal of ibuprofen (54%) and sulfamethoxazole (55%) (Banzhaf et al. 2012). However, these studies did not investigate the contribution of microbial activity in total removal of target compounds.

In the case of redox active minerals (e.g. Fe and Mn-oxyhydroxides), compound sorption may be followed by an electron transfer process resulting in the concomitant oxidation of adsorbed compound and reduction of surface-bound metal (Martin et al. 2015, Zhou et al. 2019). In the

present work, the sediment contains non-negligible amounts of Fe and Mn: 7.91mg of Fe and 0.37mg of Mn per gram of freeze-dried sediment (Table S1). According to the sequential extraction method, most of Fe and Mn may likely exist under crystallized Fe and Mn-oxyhydroxides forms (Fig. S2). Although the Fe/Mn speciation in the sediment still needs more investigation, we can suppose that the reactive minerals such as Fe and Mn-oxyhydroxides could contribute to the chemical degradation of CIP and TC.

The higher CIP adsorption as compared to TC, coupled to the higher chemical degradation of TC as compared to CIP, may be related to the binding mechanisms and kinetics as well as the redox potential of each compound. Our previous work showed that weaker binding of phenolic compounds is more energetically favorable than the surface complexation of polyfunctional carboxylated compounds (e.g. CIP), and may enable higher removal rate with minerals (Jia et al. 2021). Therefore, we suppose that interactions of TC with mineral surfaces are faster than with CIP, enabling higher oxidation rates. Furthermore, TC can also be transformed by hydrolysis at pH higher than 7 (Loftin et al. 2008). No potential by-products using LC/MS could be detected in the current study, probably because of the very low concentration of byproducts and/or their strong binding to sediments constituents. Taken together, these findings showed that the coastal sediment has a great capacity to remove both TC and CIP through adsorption and chemical degradation under our experimental conditions.

## 4. Conclusions

Our study showed that coastal sediment could contribute to affect the mobility of TC and CIP, through adsorption and degradation processes, both under static and flow-through conditions. Chemical-driven transformation accounts for the largest proportion in TC/CIP removal with respect to biological process. This is likely due to the sediment composition and characteristics. TC is less adsorbed (~20%) but more chemically transformed (~70%) in sediment than CIP (~54 % adsorption and ~36 % transformation). Greater reactivity of TC with redox-active mineral surfaces under nitrate reducing conditions may explain its higher degradation efficiency in the sediment. Dynamic flow experiments showed that simultaneous injection of CIP and TC did not alter the mobility behavior of antibiotic or nitrate with respect to the single system. Although TC and CIP strongly interacted with the sediment components through sorption and (bio)-chemical transformation, they keep their antimicrobial activities. Indeed, inhibitory effect on the nitrate reducing activity was observed, despite the absence of TC and/or CIP in

the outflow solution. This impact on the microbial growth and activities may also explain the low contribution of microbe-driven mechanism. The present work showed that assessments of the environmental risks of antibiotics must consider all physiochemical and biochemical interactions with the surrounding media, and not only their dissolved concentration in aquatic systems. These findings have, therefore, important implications in accurately predicting the fate and mobility of these emerging compounds in nature. Further investigations should focus on the assessment of the antimicrobial activities of transformation products of antibiotics in coastal sediments, and their impact on the biogeochemical cycle of nitrogen.

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## **Supporting information**

Fate and transport of tetracycline and ciprofloxacin and impact on nitrate reduction activity  
in coastal sediments from the Seine Estuary, France

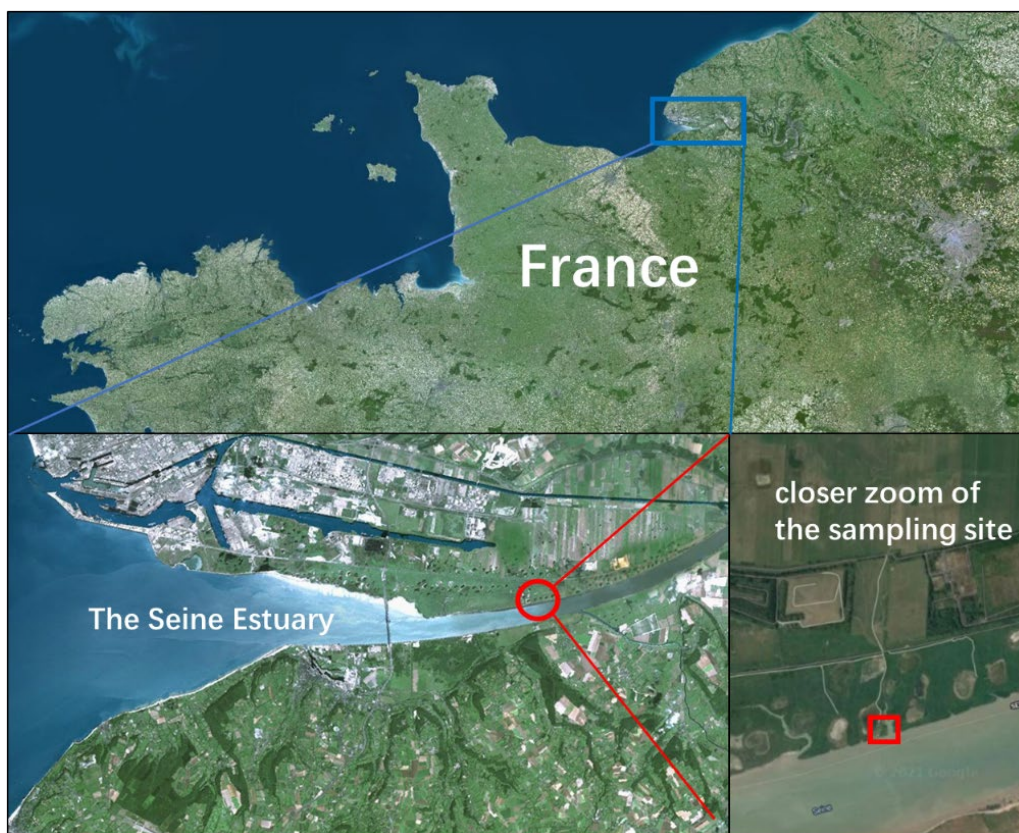


Figure S1. Sample point of coastal sediment

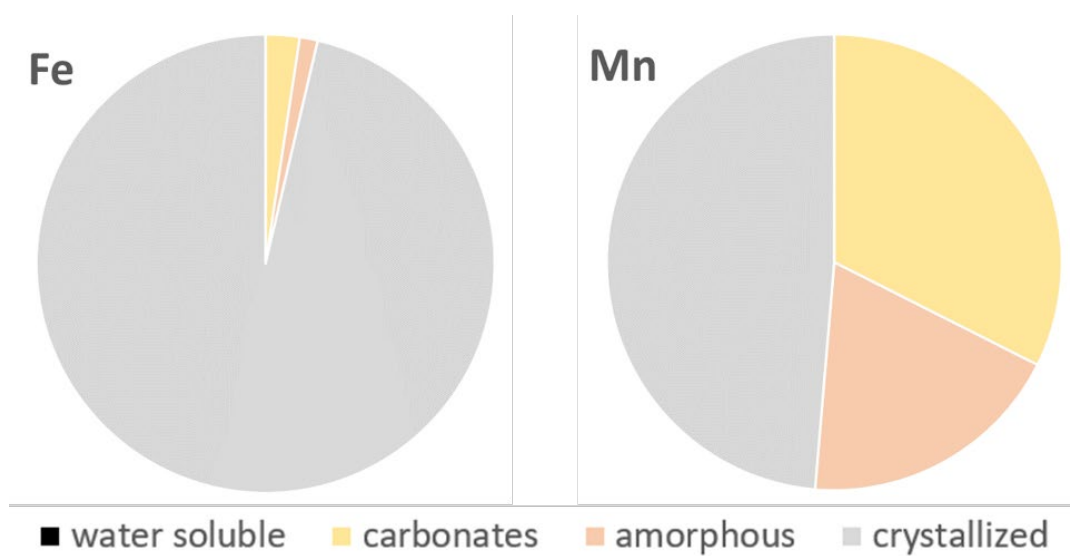


Figure S2. Sequential acid digestion results for the trace metal element Fe and Mn

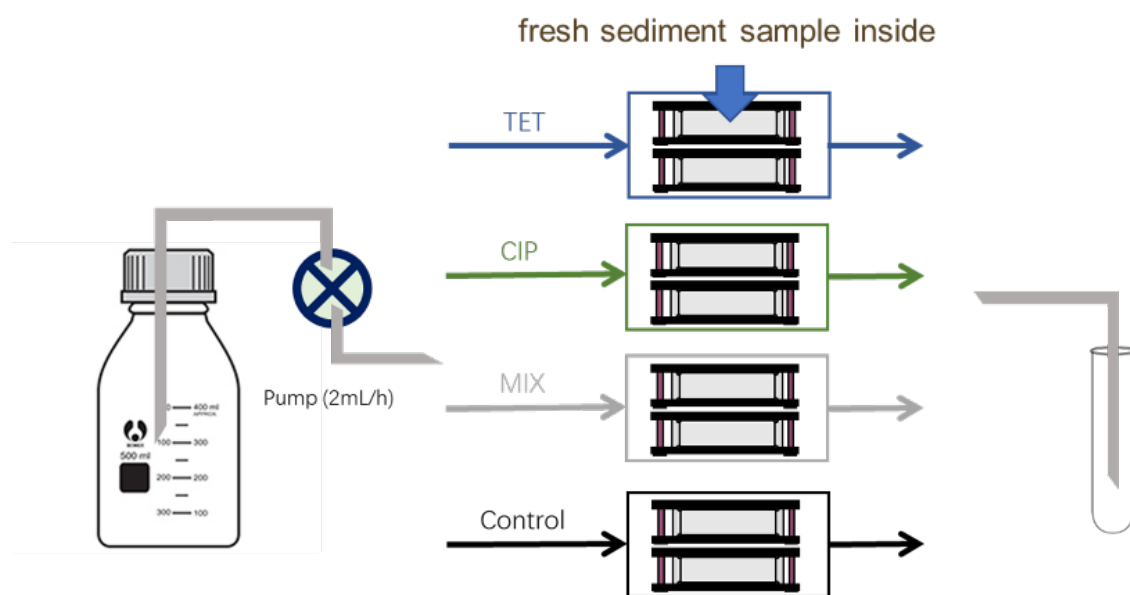


Figure S3. Scheme of the Flow-through reactors (FTR)

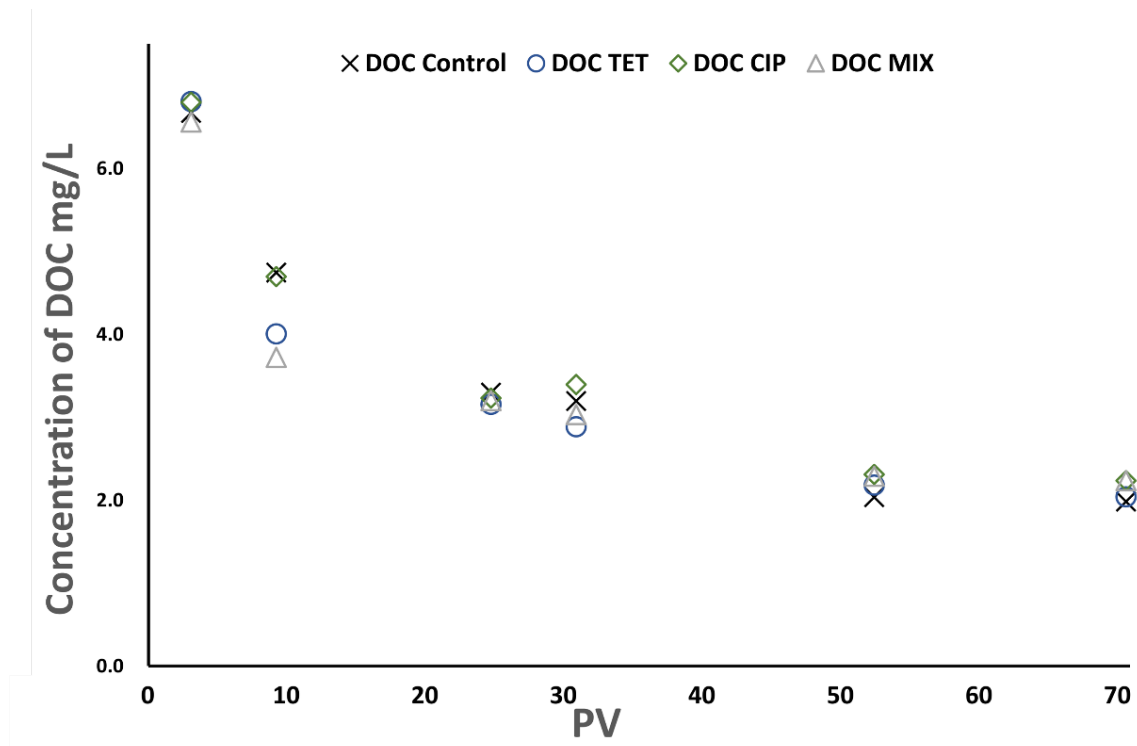


Figure S4. Concentrations of DOC against pore volume (PV) for FTR

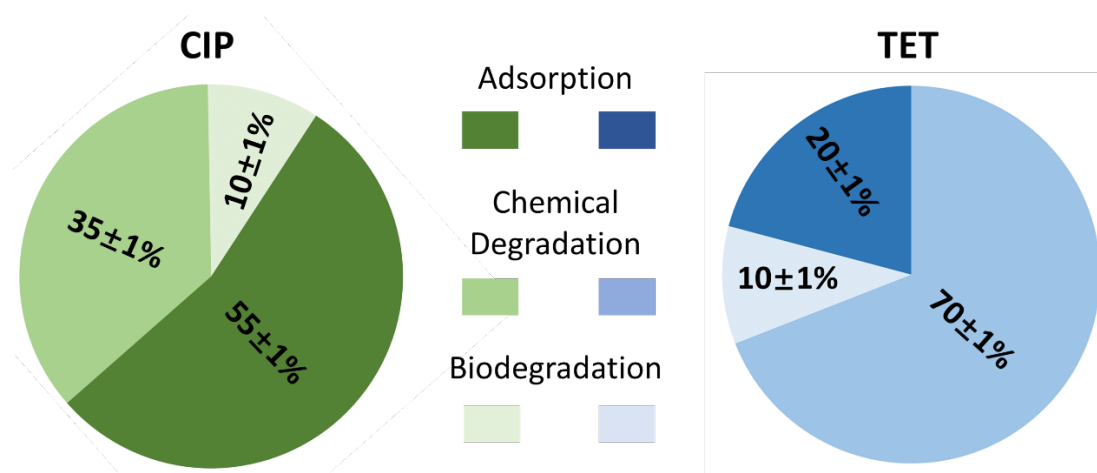
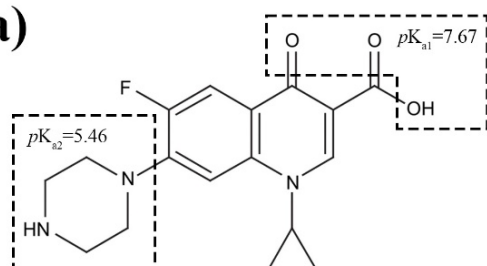
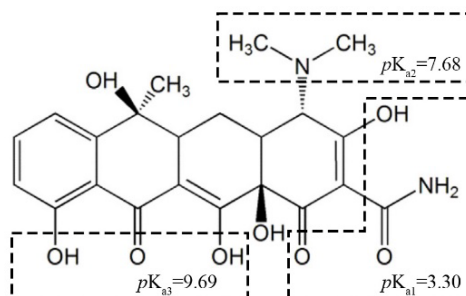


Figure S5. Contribution ratio of each individual TC (TET)/CIP removal processes (including chemical degradation, biological degradation and adsorption) in the batch of fresh sediments

**a)**

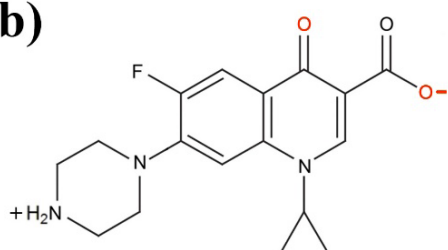


Ciprofloxacin

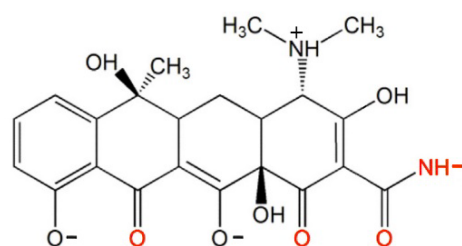


Tetracycline

**b)**



Ciprofloxacin (+/-)



Tetracycline (+/-/-)

Figure S6. a) Chemical structure and  $pK_a$  value of CIP and TC due to combinations of different groups; b) zwitterion species of CIP and TC under pH 8, binding involved atoms in red.

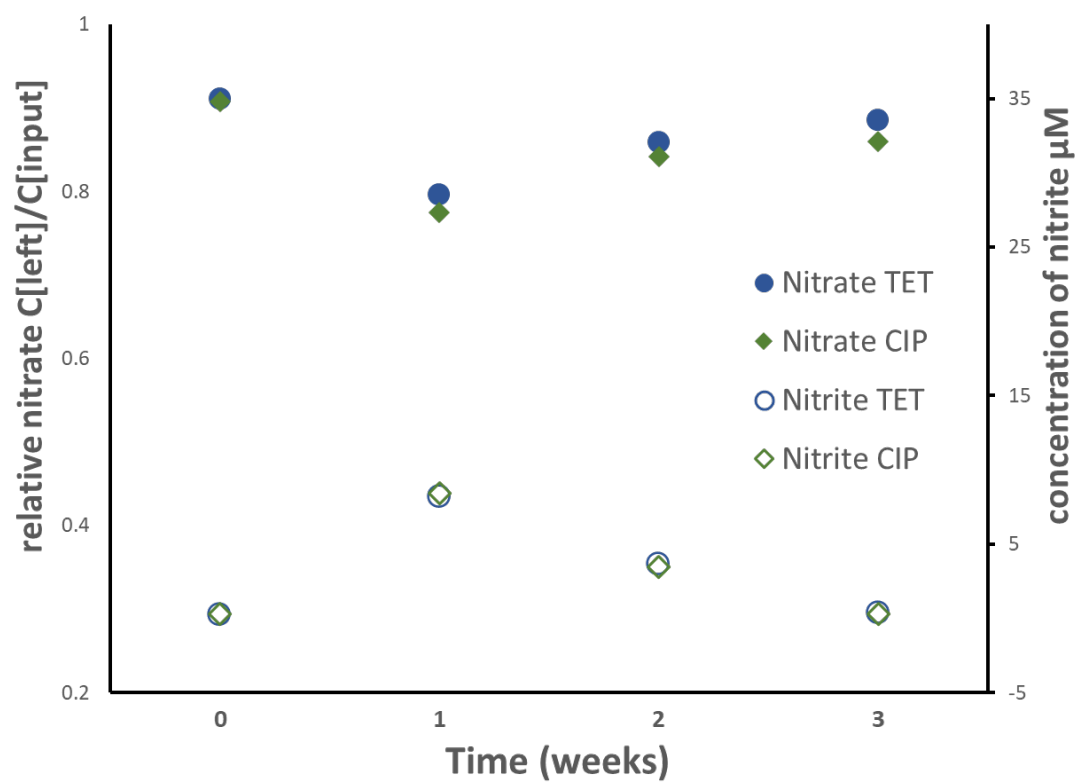


Figure S7. Concentrations of nitrate and nitrite against time for batch of fresh sediments during the 3 experimental weeks. Main Y-axis: relative nitrate concentrations for solution left to input; second Y-axis: quantified concentrations of nitrite for batch initial input and left in solution.

Table S1. Characteristics of the Seine River sediment

Parameter	Clay	Silt	Sand	TOC	TON	
Proportion %	5±1	70±1	25±1	1.49±0.1	0.13±0.01	
Parameter	Ca	Al	Mg	Fe	Mn	Zn
Proportion mg/g	84.46±4.12	4.78±0.23	4.49±0.22	7.91±0.39	0.37±0.02	0.21±0.01

Table S2. Average NO<sub>3</sub> consumption and NO<sub>2</sub> production at different PV periods in FTR

PV period	0-12PVs	20-32PVs	40-PVs
<b>NO<sub>3</sub> consumption (μM)</b>			
<b>Control</b>	1458±202	674±321	156±93
<b>TC</b>	1135±182	572±284	77±67
<b>CIP</b>	1293±282	690±235	302±74
<b>MIX</b>	1085±268	447±276	0±135
<b>NO<sub>2</sub> production (μM)</b>			
<b>Control</b>	299±190	42±28	27±3
<b>TC</b>	117±101	84±31	27±6
<b>CIP</b>	113±102	26±10	7±1
<b>MIX</b>	98±58	31±7	8±2

# **Chapter 5**

## **Conclusions and perspectives**



Antibiotics have become ubiquitous in the environment due to their widespread use in human and veterinary medicine, as well as in agriculture. These compounds can persist in sediments for extended periods, potentially impacting microbial processes and ecosystem functioning. As shown in Figure 1, my PhD thesis focuses on the effects of antibiotics on denitrification bacteria and processes in river sedimentary ecosystems, with a specific emphasis on the impact of antibiotic pollutants on microbial growth and denitrification activity. The effects of antibiotic pollutants on bacteria may be attenuated by natural minerals. The interaction between pollutants and minerals, such as manganese oxides, was investigated, with a focus on the potential for natural manganese oxides to mitigate the environmental impact of antibiotics in sedimentary systems.

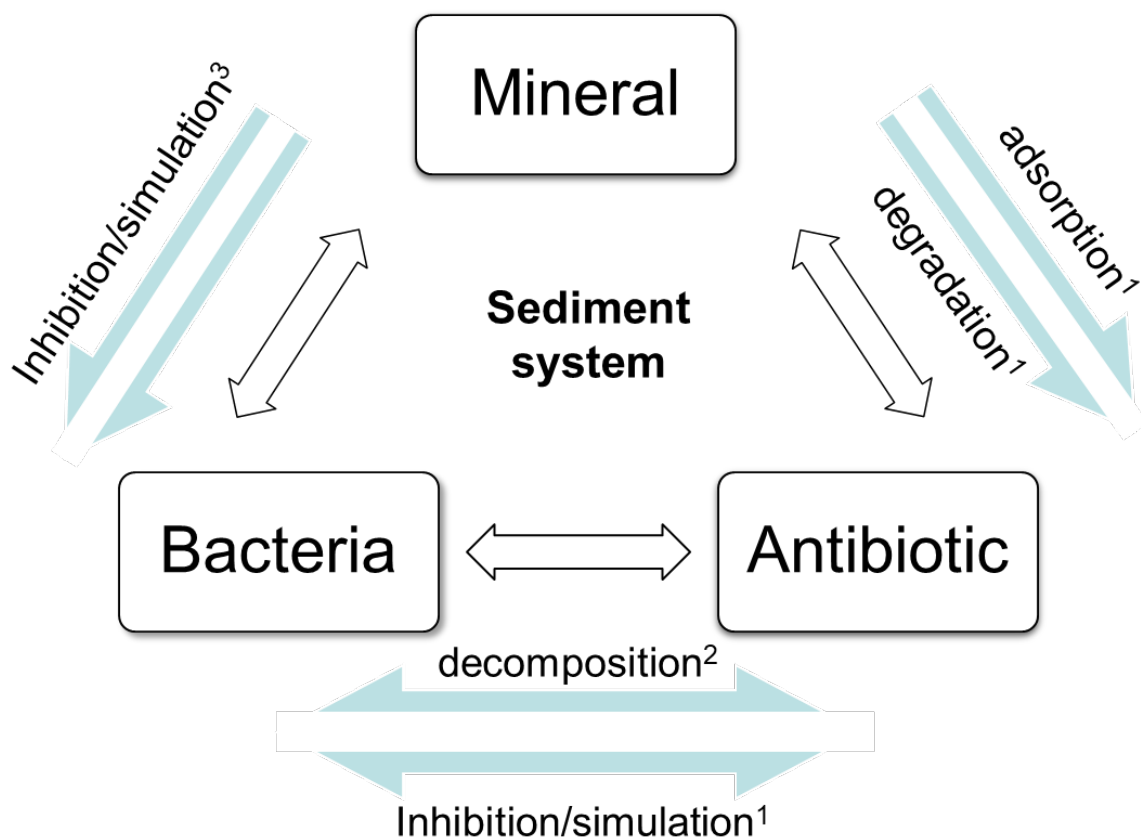


Figure 1 Possible interactions between antibiotics, minerals and bacteria in the sediment system. <sup>1</sup> means that these processes were confirmed in this thesis; <sup>2</sup> means that the process was not observed in the present work and need further determinations; <sup>3</sup> represents to future planning.

These findings improve/inspire our understanding in the followed aspects.

## 1. Interactions between antibiotics and environmental bacteria

The widespread use of antibiotics has led to their presence in the environment, including in aquatic sediments. These compounds can have significant impacts on microbial processes and ecosystem functioning. According to the results from this thesis, we could know that antibiotics can have significant inhibitions on the growth and denitrification activity of bacteria.

It was shown, in **chapter 2**, that the non-pathogenic, denitrifying bacteria *Pseudomonas veronii* was resistant towards macrolides (erythromycin and tylosin), tetracycline and the quinolone flumequine. On the other hand, *P. veronii* was susceptible towards the quinolones (ciprofloxacin and ofloxacin). Resistance towards the large lipophilic antibiotics (erythromycin and tylosin) is most likely related to the exclusionary properties of the cell envelope. In parallel antibiotic resistance genes in the genome of the denitrifier were identified via annotation through MaGe from the MicroScope platform. The genotypic antibiotic resistance of the denitrifier *P. veronii* showed the presence of several genes encoding efflux pumps (e.g. AcrAB-TolC). The presence of efflux pump genes most likely explains resistance towards the other tested antibiotics. The experimental results of *P. veronii* under denitrification conditions demonstrate that different antibiotics have varying negative impacts on denitrification capabilities, with the nitrite reduction process being more susceptible to antibiotic impact than the nitrate reduction process.

The release of antibiotics into the environment is a major concern due to their potential impact on human health and the environment. Antibiotic residues can be found in various environmental matrices, such as soil, water, and sediment, and their degradation products may persist for long periods of time. Therefore, it is important to investigate the potential effects of antibiotic residues and their degradation products on environmental microorganisms, including those in sediment communities.

Among the various antibiotic classes, tetracycline (TC) and ciprofloxacin (CIP) are among the most frequently detected in the environment due to their high usage and persistence. TC is a broad-spectrum antibiotic that inhibits bacterial protein synthesis by binding to the ribosome, while CIP belongs to the fluoroquinolone class and inhibits bacterial DNA synthesis by targeting DNA gyrase and topoisomerase IV. The persistence and frequent detection of TC and CIP in environmental matrices, such as water, sediment, and soil, raise concerns about their potential impacts on microbial communities and ecosystem functioning. Moreover, antibiotic residues in the environment could be present in different forms, including partially degraded

byproducts, which could exhibit different properties and activities compared to the parent compound.

In addition to the effect of antibiotics on growth and functioning of denitrifiers, **Chapter 3** investigated the effects of CIP and its byproducts on both growth and activity on a denitrifying species isolated from river sediment. Our results showed that 4 oxidation byproducts of CIP were identified during the reaction with birnessite at a pH of 5. Although lower than CIP, one of the byproducts remained its antimicrobial activity in inhibiting the growth of the denitrifier *Pseudomonas veronii*. Batch experiments showed that the nitrite reduction rate was lowered when CIP was added, while nitrate reduction rates were kept at a same level. In the case of TC and its byproducts, though the growth of *P. veronii* decreased, both nitrate and nitrite reduction activities of *P. veronii* were not essentially inhibited. This could be due to the fact that activities of nitrite reductases are easily affected by antibiotic contaminants compared to nitrate reductases.

In the controlled environment of a pure culture, researchers can explore the effects of specific variables on the growth and behavior of the bacterium. However, in natural environments like sediment, microbial communities are highly diverse and complex, with multiple species interacting in intricate ways. This complexity makes it challenging to isolate and study the impact of individual factors on microbial communities. Nonetheless, studying microbial communities in sediment provides critical insights into the dynamics and ecology of microorganisms in their natural habitat. By examining the interactions between different microorganisms and their environment, we can better understand the role of microorganisms in ecosystem functioning and the broader impacts of environmental changes on microbial communities.

Thus, the effects of antibiotics on denitrifiers under the environmental conditions were studied in **Chapter 4**. A certain alteration of the nitrate reduction and nitrite production during the first period of breakthrough was observed, indicating that the presence TC and CIP could inhibit microbial activity, since less nitrate to nitrite conversion was achieved with respect to the control test. This may be due to their negative effects on microbial growth and activities.

These findings improve our understanding of the antimicrobial activity and toxicity of antibiotics and their oxidation byproducts, especially against environmental bacteria.

However, it is imperative to understand the underlying mechanisms involved in this interaction. Further research should focus on a biological explanation. In particular, the role of genes and

enzymes in the antibiotic-mediated suppression of bacterial denitrification needs to be elucidated.

In recent years, quantitative polymerase chain reaction (qPCR) has emerged as a useful tool for studying the gene expression and enzymatic activity of microorganisms, including denitrifying bacteria (Philippot et al. 2007). By using qPCR, researchers can investigate the expression of key genes involved in denitrification, such as *nirS*, *nirK*, and *nosZ* (Gruntzig et al. 2001, Henry et al. 2004). These genes encode enzymes that catalyze specific steps of the denitrification process, including the reduction of nitrite to nitric oxide and the conversion of nitrous oxide to nitrogen gas (Philippot 2002). By monitoring the expression levels of these genes, researchers can gain insights into the effects of antibiotics on the enzymatic activity of denitrifying bacteria. It could be a supplement to **chapter 2** using qPCR to determine the expression of certain genes, to explain the inhibition of different antibiotics on growth and activity of *P. veronii*. Meanwhile, based on our work in **chapter 3**, the genetic changes of environmental bacteria caused by antibiotic byproducts would be a new orientation, which will also benefit from qPCR technology. In addition to gene expression, qPCR can also be used to quantify the abundance of specific bacterial populations in environmental samples. By comparing the relative abundances of denitrifying bacteria in different samples, the impact of antibiotics on the composition of bacterial communities in sediment could be better evaluated in **chapter 4**. By combining qPCR with other techniques such as metagenomics and proteomics, we can gain a comprehensive understanding of the changes on bacterial denitrification and give biological explanations.

## **2. Interactions between antibiotics and natural minerals**

Antibiotics can enter the environment through various pathways, such as wastewater treatment plants, agricultural runoff, and animal manure. Once in the environment, antibiotics can interact with environmental minerals, which can affect their bioavailability and biodegradability, as well as their potential to select for antibiotic-resistant bacteria. Two representative antibiotics, ciprofloxacin (CIP) and tetracycline (TC), were used in the following studies.

The mineral induced antibiotic degradation was studied in **Chapter 3**, through the investigation of the oxidation processes of CIP and TC induced by birnessite under acidic denitrifying conditions, and the proposal of certain degradation pathways. The results showed that

birnessite played a significant role in the oxidation of CIP and TC, and that the oxidation was dependent on the pH of the reaction solution. The degradation pathway of CIP involved a series of steps including the formation of intermediate products, followed by the cleavage of the piperazine ring. For TC, the main degradation pathway involved the formation of various intermediates, and ultimately degradation into simpler compounds. The proposed degradation pathways were supported by the identification of various intermediates and degradation products using techniques such as HPLC and LC-MS. These results provide important insights into the mechanisms of antibiotic degradation by minerals in the environment.

Moreover, **Chapter 4** improved our understanding of the transport of TC and CIP in natural mineral containing media, a sediment sampled from north site in Seine River, under nitrate reducing conditions. Dynamic flow experiments showed that the sediment has a large capacity to remove TC or CIP, which can be ascribed to sorption to sediment constituents and/or (bio)-chemical transformation. When CIP and TC were simultaneously injected, no significant difference in the shape of breakthrough curves was observed. Batch kinetics experiments conducted over 3 weeks with freeze-dried vs fresh sediments showed that abiotic degradation (mineral redox reactions) is the major controlling process, compared to biotic or microbe-driven process. The abiotic degradation can be attributed to metal oxides present in the sediment, as iron and manganese oxides were detected via sequential extraction methods.

These findings have important implications in the assessment of transport and impact of antibiotics and their byproducts in coastal sediments in a multiple contaminated system. Meanwhile, our findings also suggest that metal oxides, particularly manganese oxides, can be a potential tool for reducing the environmental impact of antibiotics in sediments.

However, there are still some questions that need to be addressed in future research. One important area of investigation is the effects of other minerals on a wider range of antibiotics. While this study focused on the effects of birnessite on CIP and TC, other minerals present in sediment may have different effects on these antibiotics or on other types of antibiotics altogether. Thus, further studies could explore the effects of different minerals on a variety of antibiotics, to provide a more complete understanding of how these compounds interact in sediment environments.

Another important area for future research is the use of additional testing methods to analyze the mineral composition of sediment. While X-ray diffraction is a common technique used to identify minerals, it is limited by its inability to detect amorphous phases or minerals that are

present in very low concentrations. Other techniques, such as scanning electron microscopy, transmission electron microscopy, and Fourier transform infrared spectroscopy, may provide more detailed information about the composition and structure of minerals in sediment, enabling a more nuanced understanding of their interactions with antibiotics.

Finally, the development of new methods to identify antibiotic degradation products in sediment environments could provide valuable information about the fate and transport of these compounds. While this study focused on the degradation of CIP and TC in the presence of birnessite, other degradation products may be present in sediment and could have different toxicological effects on the ecosystem. The development of sensitive and specific methods to identify and quantify these products could help to inform environmental risk assessments and guide the development of more effective strategies for mitigating the impacts of antibiotic pollution.

### **3. Minerals, antibiotics and denitrifiers: the sediment system**

Studying the interactions among antibiotics, minerals, and denitrifying bacteria is crucial to developing effective strategies for mitigating the environmental risks, as it can provide insight into understanding the fate and transport of antibiotics in natural systems and their potential impacts on microbial communities and nutrient cycling. The sediment system is the suitable environment to study the interaction of antibiotics, minerals, and denitrifying bacteria. Minerals, as important components of sediment, can interact with antibiotics and influence their transport in the environment. On the other hand, denitrifying bacteria in sediment play an essential role in the global nitrogen cycle, and their activities can be influenced by the presence of antibiotics and minerals.

As a simulation of the natural environment, **Chapter 4** investigated the fate of antibiotics in a natural sediment sample using Flow-Through Reactors (FTRs). FTRs can be designed to simulate specific environmental conditions, such as temperature, flow rate, and nutrient levels, which allows to study how different variables affect the fate and transport of contaminants. In this work, FTRs allowed to study antibiotic contaminants under continuous supplementary in situ, which provides ideal conditions like saturated sediments. The results showed that the sediment has a significant capacity to remove CIP and TC, mainly due to sorption to sediment constituents and chemical transformation (degradation). In parallel, less nitrate to nitrite conversion was achieved compared to the control test, suggesting that the antibiotics had

negative impacts on microbial activity. This study provides valuable insights into the fate of antibiotics in sediment systems by evaluating the changes of nitrate and nitrite content.

In future research, it may be useful to investigate the effect of other minerals on the fate of different types of antibiotics, as well as to analyze the mineral composition of sediments through additional testing methods. Furthermore, developing new methods to identify antibiotic degradation byproducts in sediment environments could also provide valuable insights. In addition to the aforementioned three points, it is also necessary to investigate other microbial activities, such as nitrification and anaerobic ammonium oxidation, in the context of combining antibiotics and minerals. These activities can potentially play important roles in the fate and transport of antibiotics in sediment systems. For instance, nitrifying bacteria can oxidize ammonium and produce nitrite, which can further react with metal oxides and affect the sorption and degradation of antibiotics. Anaerobic ammonium oxidation, on the other hand, can remove ammonium and nitrite from sediment pore water and release nitrogen gas, which may affect the nitrogen cycle and the overall microbial community. Therefore, future studies should also consider the interactions between antibiotics, minerals, and other microbial activities to obtain a more comprehensive understanding of the fate and impact of antibiotics in sediment systems.

In summary, while our study has revealed the impact of antibiotics and their mineral induced byproducts on denitrifying bacteria, there is still much to be learned about the direct effect of minerals/mineral-bound antibiotics on bacterial growth and metabolism. By developing a better understanding of antibiotic-mineral-microbe interactions, we can improve our ability to predict and manage the cycling of nitrogen and other essential nutrients in the environment, which has important implications for sustainable agriculture, water quality management, and global biogeochemical cycles.

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# Résumé

La contamination environnementale par les antibiotiques est de plus en plus préoccupante en raison de leur capacité à sélectionner des bactéries résistantes aux antibiotiques. Du fait de leur action spécifique sur les bactéries, les antibiotiques peuvent entraîner des perturbations des cycles biogéochimiques qui sont régis par les activités bactériennes et notamment le cycle de l'azote. L'impact des antibiotiques sur les organismes en charge de la dénitrification (une étape clé du cycle de l'azote) dépend de la disponibilité, de la forme et de la spéciation des antibiotiques dans les sédiments, qui dépendent de la composition du sédiment, notamment de ses minéraux. Les oxydes métalliques présents dans les sédiments pourraient interférer avec les antibiotiques par sorption et/ou dégradation. Par ailleurs, la présence de minéraux dans les sédiments peut également avoir des implications importantes pour les processus microbiens, y compris la dénitrification. Dans les sédiments où les antibiotiques peuvent s'accumuler et où le temps de contact entre les bactéries et les antibiotiques est élevé, l'effet des antibiotiques sur les bactéries bénéfiques pour l'environnement comme les dénitrifiants doit être étudié, ainsi que les interactions avec les phases minérales et organiques du sédiment. Cela souligne les interactions complexes entre les minéraux, les micro-organismes et les contaminants anthropiques dans les sédiments naturels. Comprendre ces interactions est essentiel pour développer des stratégies efficaces pour gérer et atténuer l'impact de la pollution sur les environnements sédimentaires.

Ces interactions entre les antibiotiques, les minéraux et l'activité bactérienne sont à la base de mon sujet de thèse intitulé « transfert des antibiotiques aux interfaces minérales : impacts sur la dénitrification dans les systèmes sédimentaires aquatiques ». Ma thèse se compose des chapitres suivants :

Dans le chapitre 2, sont étudiés les effets de différents antibiotiques sur la croissance et l'activité dénitrifiante d'une bactérie environnementale. Le but de ce chapitre est de répondre aux questions suivantes :

- Les gènes de résistance aux antibiotiques sont-ils présents dans une bactérie dénitrifiante environnementale non pathogène ?
- Les antibiotiques affectent-ils la croissance et l'activité dénitrifiante de cette espèce ?

Dans le chapitre 3, sont étudiés les effets des antibiotiques et de leurs produits de dégradation sur l'activité de dénitrification d'une bactérie dénitrifiante environnementale *P. veronii*. Le but de ce chapitre est de répondre aux questions suivantes :

- Comment se transforment les antibiotiques tétracycline (TC) et de ciprofloxacine (CIP) avec l'oxyde de manganèse, la birnessite ? Quels sont les sous-produits ?
- Est-ce que les sous-produits de TC et CIP affectent la croissance et l'activité de *P. veronii* ?

Le chapitre 4 décrit le transport et le devenir des molécules de TC et de la CIP dans des

sédiments côtiers complexes afin de répondre aux questions suivantes :

- Les sédiments côtiers peuvent-ils agir comme piège pour la TC et la CIP ?
- Des transformations ou des dégradations de la TC et de la CIP peuvent-elles se produire dans les sédiments côtiers ?
- Les bactéries contribuent-elles à l'élimination de la TC et de la CIP dans les sédiments ?

En se basant sur les questions mentionnées ci-dessus, à travers une revue de la littérature et des recherches expérimentales, ma thèse a principalement permis d'obtenir les résultats suivants.

#### - **L'interaction entre les antibiotiques et les bactéries environnementales**

Selon les résultats de cette thèse, nous pouvons constater que les antibiotiques peuvent entraîner des inhibitions significatives sur la croissance et l'activité de dénitrification des bactéries. Il a été démontré dans le chapitre 2 que la bactérie dénitrifiante *Pseudomonas veronii* était résistante aux macrolides (érythromycine et tylosine), à la tétracycline et à la quinolone fluméquine. En revanche, *P. veronii* était sensible à deux autres quinolones (ciprofloxacine et ofloxacine). La résistance aux antibiotiques lipophiles de grande taille (érythromycine et tylosine) est très probablement liée aux propriétés d'exclusion de l'enveloppe cellulaire. En parallèle, des gènes de résistance aux antibiotiques ont été identifiés dans le génome de la bactérie dénitrifiante via MaGe de la plateforme MicroScope. La résistance aux antibiotiques génotypique de *P. veronii* a montré la présence de plusieurs gènes codant pour des pompes à efflux (par exemple AcrAB-TolC). La présence de gènes de pompes à efflux explique très probablement la résistance aux autres antibiotiques testés. Les résultats montrent que différents antibiotiques ont des impacts négatifs variables sur les capacités de dénitrification. De plus, le processus de réduction du nitrite est plus sensible à l'impact des antibiotiques que le processus de réduction du nitrate.

Parmi les différentes classes d'antibiotiques, la tétracycline (TC) et la ciprofloxacine (CIP) sont les plus fréquemment détectées dans l'environnement en raison de leur forte utilisation et de leur persistance. Les résidus d'antibiotiques dans l'environnement peuvent être présents sous différentes formes, notamment sous forme de produits de dégradation partielle, qui pourraient présenter des propriétés et des activités différentes par rapport au composé parent. En plus de l'effet des antibiotiques sur la croissance et le fonctionnement des dénitrifiants, le chapitre 3 a étudié les effets de la CIP et de ses produits de dégradation sur la croissance et l'activité d'une espèce dénitrifiante. Les résultats de cette thèse montrent que quatre produits d'oxydation de la CIP ont été identifiés lors de la réaction avec de la birnessite à un pH de 5. Bien qu'inférieure à celui de la CIP, l'activité antimicrobienne de l'un des produits de dégradation a été conservé : il inhibait la croissance du dénitrifiant *Pseudomonas veronii*. Des expériences en batch ont montré que le taux de réduction des nitrites était réduit lorsque la CIP était ajoutée, tandis que les taux de réduction des nitrates étaient maintenus à un niveau similaire. Dans le cas de la TC et de ses produits de dégradation, bien que la croissance de *P.*

*veronii* ait diminué, les activités de réduction des nitrates et des nitrites de *P. veronii* n'ont pas été inhibées. Cela pourrait s'expliquer par le fait que les activités des nitrite réductases sont plus facilement affectées par les contaminants antibiotiques que les réductases de nitrate.

Contrairement aux expérimentations avec des souches pures, les environnements naturels comme les sédiments, possède une grande diversité microbienne, avec des communautés microbiennes interagissant de manière complexe. En examinant les interactions entre différents micro-organismes et leur environnement, nous pouvons mieux comprendre le rôle des micro-organismes dans le fonctionnement des écosystèmes et les impacts des changements environnementaux sur les communautés microbiennes. Ainsi, les effets des antibiotiques sur les dénitrifiants en conditions environnementales ont été étudiés dans le chapitre 4. Une certaine altération de la réduction de nitrate et de la production de nitrite pendant la première période a été observée, indiquant que la présence de TC et de CIP pourrait inhiber l'activité microbienne, car moins de conversion de nitrate en nitrite a été réalisée par rapport au contrôle. Cela peut être dû à leurs effets négatifs sur la croissance et les activités microbiennes. Ces résultats améliorent notre compréhension de la toxicité des antibiotiques et de leurs produits d'oxydation sur l'activité antimicrobienne, en particulier envers les bactéries environnementales.

#### - **L'interaction entre les antibiotiques et les minéraux naturels**

L'étude de la dégradation des antibiotiques induite par les minéraux a été effectuée dans le chapitre 3, par le biais de l'investigation des processus d'oxydation de CIP et TC induits par la birnessite dans des conditions de dénitrification, ainsi que la proposition de certaines voies de dégradation. Les résultats ont montré que la birnessite jouait un rôle significatif dans l'oxydation de CIP et TC, et que l'oxydation dépendait du pH de la solution de réaction. La voie de dégradation de CIP impliquait une série d'étapes, y compris la formation de produits intermédiaires, suivie de la rupture de l'anneau de pipérazine. Pour TC, la principale voie de dégradation impliquait la formation de divers intermédiaires, et finalement la dégradation en composés plus simples. Les voies de dégradation proposées ont été soutenues par l'identification de divers intermédiaires et produits de dégradation en utilisant des techniques telles que HPLC et LC-MS. Ces résultats fournissent des informations importantes sur les mécanismes de dégradation des antibiotiques par les minéraux dans l'environnement.

De plus, le chapitre 4 a amélioré notre compréhension du transport de TC et CIP dans un sédiment de la Seine, contenant des minéraux dans des conditions de réduction de nitrate. Des expériences de réacteurs en flux continu ont montré que le sédiment avait une grande capacité à éliminer TC ou CIP, ce qui peut être attribué à la sorption aux constituants du sédiment et/ou à la transformation (bio)-chimique. Lorsque CIP et TC ont été injectés simultanément, aucune différence significative n'a été observée. Des expériences de cinétique en batchs menées sur 3 semaines avec des sédiments lyophilisés versus frais ont montré que la dégradation abiotique (réactions de rédox minérales) est le processus majeur par rapport au processus biotique ou microbien. La dégradation abiotique peut être attribuée aux oxydes métalliques présents dans le sédiment, car les oxydes de fer et de manganèse ont été détectés

par des méthodes d'extraction séquentielle.

- **Minéraux, antibiotiques et dénitrifiants : le système sédimentaire**

L'étude des interactions entre les antibiotiques, les minéraux et les bactéries dénitrifiantes est cruciale pour développer des stratégies efficaces de réduction des risques environnementaux, car elle peut fournir des informations sur le devenir et le transport des antibiotiques dans les systèmes naturels et leurs impacts potentiels sur les communautés microbiennes et le cycle des nutriments. Le système de sédiments est l'environnement adapté pour étudier l'interaction des antibiotiques, des minéraux et des bactéries dénitrifiantes. Les minéraux, en tant que composants importants des sédiments, peuvent interagir avec les antibiotiques et influencer leur transport dans l'environnement. D'autre part, les bactéries dénitrifiantes dans les sédiments jouent un rôle essentiel dans le cycle de l'azote global, et leurs activités peuvent être influencées par la présence d'antibiotiques et de minéraux.

En développant une meilleure compréhension des interactions antibiotique-minéral-microbe, nous pouvons améliorer notre capacité à prédire et gérer le cycle de l'azote et d'autres nutriments essentiels dans l'environnement, ce qui a des implications importantes pour l'agriculture durable, la gestion de la qualité de l'eau et les cycles biogéochimiques mondiaux.

**Titre :** Antibiotiques dans l'environnement : impact des oxydes métalliques et effet sur la dénitrification

**Mots clés :** Antibiotiques, Dénitrification, Birnessite, Milieu sédimentaire, Transport, Dégradation.

**Résumé :** Les antibiotiques sont devenus omniprésents dans l'environnement en raison de leur utilisation généralisée en médecine humaine et vétérinaire, ainsi qu'en agriculture. Ces composés peuvent persister dans les sédiments pendant de longues périodes, impactant potentiellement les processus microbiens et le fonctionnement des écosystèmes. Cependant, les effets des polluants antibiotiques sur les bactéries peuvent être atténués par des minéraux naturels, tels que les oxydes de manganèse. Par conséquent, cette thèse se concentre sur les effets des antibiotiques sur les bactéries et les processus de dénitrification dans les écosystèmes sédimentaires fluviaux, en mettant l'accent sur l'interaction entre les antibiotiques, les minéraux et les bactéries. Dans la première partie, la résistance aux antibiotiques d'une bactérie dénitrifiante non pathogène, *Pseudomonas veronii*, a été étudiée ainsi que l'effet de plusieurs

antibiotiques représentatifs sur son activité dénitrifiante. Dans la deuxième partie, les mécanismes de transformation de la ciprofloxacine et de la tétracycline par un minéral commun du sol, la birnessite ( $MnO_2$ ), ont été étudiés pour déterminer les effets des sous-produits de transformation sur la croissance et l'activité de la même bactérie dénitrifiante. La troisième partie s'est concentrée sur le transport de la ciprofloxacine et de la tétracycline dans les sédiments de l'estuaire de la Seine (France), dans des conditions de réduction de nitrate. Ces résultats améliorent notre compréhension de l'activité antimicrobienne et de la toxicité des antibiotiques et de leurs sous-produits d'oxydation, en particulier contre les bactéries environnementales, ce qui a des implications importantes pour l'agriculture durable, la gestion de la qualité de l'eau et des sédiments, et les cycles biogéochimiques globaux.

**Title :** Antibiotics in the environment: impact of metal oxides and effect on denitrification

**Keywords :** Antibiotics, Denitrification, Minerals, Sediment environment, Transport, Degradation.

**Abstract :** Antibiotics have become ubiquitous in the environment due to their widespread use in human and veterinary medicine, as well as in agriculture. These compounds can persist in sediments for extended periods, potentially impacting microbial processes and ecosystem functioning. However, the effects of antibiotic pollutants on bacteria may be attenuated by natural minerals, such as manganese oxides. Therefore, this thesis focuses on the effects of antibiotics on denitrification bacteria and processes in river sedimentary ecosystems, with a specific emphasis on the interaction between antibiotics, minerals and bacteria. In the first part, the antibiotic resistance in a non-pathogenic denitrifying bacterium *Pseudomonas veronii* was investigated, compared with the effects of several

representative antibiotics on denitrifying activity. In the second part, the mechanisms of transformation of ciprofloxacin and tetracycline by a common redox soil mineral, birnessite ( $MnO_2$ ), were first studied, and then the effects of transformation byproducts on both growth and activity on a denitrifying isolate were determined. The third part focused on the transport of ciprofloxacin and tetracycline in sediments from the Seine Estuary (France), under nitrate reducing conditions. These findings improve our understanding of the antimicrobial activity and toxicity of antibiotics and their oxidation by-products especially against environmental bacteria, which has important implications for sustainable agriculture, quality management of the sediment, and global biogeochemical cycles.